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(54) Title: ANTIVIRAL COMPOSITIONS AND METHODS OF USE

(57) **Abstract:** Purified antiviral compounds, pharmaceutical formulations containing the compounds, and methods of use of the compounds are provided. The compositions of the invention are isolated antiviral components from plant extracts derived from, for example, *Salvia milriorrhiza*, that find use in the treatment of viral infections, such as by inhibiting the activity of viral integrase. Methods for isolation and purification of the antiviral compounds are additionally provided.

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ANTIVIRAL COMPOSITIONS AND METHODS OF USE

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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FIELD OF THE INVENTION

The present invention relates to compositions and methods for the prevention and treatment of viral infections, particularly retroviral infections.

BACKGROUND OF THE INVENTION

Viruses consist of either double-stranded or single-stranded DNA or RNA enclosed in a protein coat, called a capsid. Some viruses also possess a lipoprotein envelope that, like the capsid, may contain antigenic proteins. Since viruses have no metabolic machinery of their own, they usurp the machinery of their host cell which, depending on the virus, may be a plant, bacterium, or animal cell.

A viral infection begins when a virion comes into contact with a host cell and attaches, or adsorbs, to it. The viral DNA or RNA then crosses the plasma membrane into the cytoplasm and eventually enter into the nucleus. In the case of retrovirus, the viral RNA is reverse transcribed into DNA. Viral DNA is then integrated into the chromosomal DNA of the infected cell. Integration is mediated by an integration protein, integrase. All integrated proviruses are required for the subsequent transcription process which is acted upon by the host cell transcription factors. The integrated DNA is transcribed by the cell's own machinery into mRNA, or replicated and becomes enclosed in a virion. For retrovirus, the integrated DNA is transcribed into RNA that either acts as mRNA or become enclosed in a virion. This completes the virus life cycle.

In the past decade, the emergence of human immunodeficiency virus type 1 (HIV-1) as an important human pathogen has led to a resurgence of scientific interest

in retroviruses. HIV-1 is the primary etiologic agent of AIDS, a fatal disease that results from the gradual destruction of the helper T-cell population in infected individuals. The importance of HIV-1 as a human pathogen has led to its being the major focus of research into lentivirus replication and gene regulation. Indeed, HIV-1 may be viewed as the prototype of not only the lentivirus subgroup but also, more broadly, complex retroviruses in general.

There are an estimated 650,000 to 900,000 people currently living with HIV in the United States, with approximately 40,000 new HIV infections occurring here every year. As of June 1999, 711,344 AIDS cases have been reported in the United States. Since the beginning of the epidemic, 420,201 AIDS deaths have been reported. The scale of the AIDS epidemic demands the development of efficient and affordable AIDS therapeutics.

While HIV-1 relies heavily on the cellular host enzymes for many of the steps required in its replication, the virus carries in its genome the genetic information that leads to the synthesis of its unique retroviral enzymes, such as the three enzymes encoded by its *pol* gene: reverse transcriptase, proteases, and integrase. Effective antiviral agents must inhibit virus-specific replicative events or preferentially inhibit virus-directed rather than host cell-directed nucleic acid or protein synthesis. To date, of the numerous compounds that have already been identified and approved for marketing by the FDA for HIV, only drugs inhibiting the activities of reverse transcriptase and protease inhibitors have been identified. The first drug to be introduced was suramin, a reverse transcriptase inhibitor. Subsequently, AZT and other compounds (zalciabine (ddC), didanosine (ddl), compound Q, ritonavir, etc.) have also been found to possess anti-HIV activity *in vitro*. Specifically, AZT was approved by the FDA in 1987.

Even though the current therapeutic agents are effective in inhibiting the enzymatic activity which is essential for the viral life cycle, the small fraction of remaining viruses unfortunately mutate and continue to replicate even in the presence of these drugs. High rates of replication, viral sequence mutation, and rapid turnover of the viral population are typical traits of retroviruses. These traits are even more

striking in the case of HIV-1. As result, these drugs show little long term benefits in terms of a complete treatment or prevention of HIV-infection. Recent studies have demonstrated that combinatorial therapy against reverse transcriptase (RT) and protease can eliminate a majority of the HIV viruses in T lymphocytes. There is, therefore, need for additional therapeutic agents to be added to the treatment cocktail for viral infections, particularly retroviral infections.

The viral integrase catalyses the integration of the viral DNA into the host DNA, which is an essential step in the viral life cycle. There is no know human homologue to this enzyme and therefore potential inhibitors could be both efficacious and non-toxic. However, drugs targeting integrase have been slow to emerge because of the lack of structural information on this poorly soluble protein. Current search on integrase inhibitor has relied more on empirical testing than on drug design.

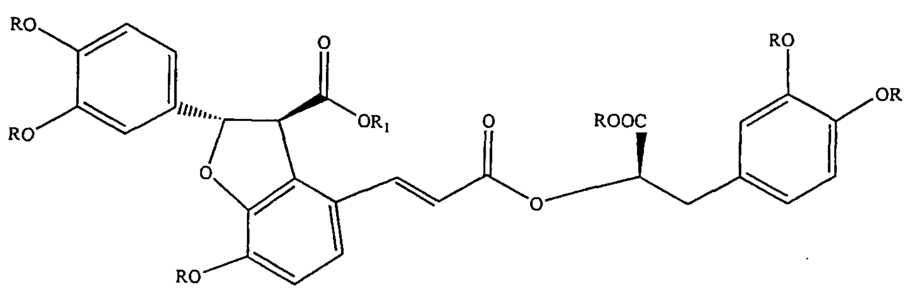
Salvia miltiorrhiza is a traditional Chinese medicinal herb for treatment of cardiovascular and hepatic diseases. Extracts from *S. miltiorrhiza* and its related species exhibit anti-viral and antioxidant activities that are health beneficial. See Meng et al. (1992) *Chung Kuo Chung Hsi I Chieh Ho Tsa Chih* 12, 345-347, 324-35; Xiong (1993) *Chung Kuo Chung Hsi I Chieh Ho Tsa Chih* 13, 33-35, 516-517; U.S. Pat. No. 5,178,865; U.S. Pat. No. 5,411,733; U.S. Pat. No. 6,043,276; International PCT Application 98/24460; Chinese Patent Application Nos. 1,192,922 and 1,192,918. Antiviral agents active against herpes, polio, measles, varicellazoster, cytomegalovirus, DNA viruses and RNA viruses have been described which contain at least one crude drug from the root of *S. miltiorrhiza* Bunge (See European Patent No. 0 568 001 A2). Seven phenolic compounds isolated from the aqueous extract of *S. miltiorrhiza* demonstrate a strong protective action against peroxidative damage to liver microsomes, hepatocytes, or erythrocytes (See Liu, *et al.*, 1992, *Biochem. Pharmacol.* 43, 147-1952). Lithospermic acid B was identified as an active component in an extract of *Salvia miltiorrhiza radix* that was shown to exhibit endothelium-dependent vasodilation in the aorta and may be useful in the treatment of hypertension (See Kamata, *et al.*, 1993, *Gen. Pharmacol.* 24, 977-981). The therapeutic effect of these extract has been attributed in part to the ability of the plant

to accumulate active compounds such as transhinones and phenolic compounds.

Therefore, there remains a need in the art for the identification of additional compounds capable of treating viral infections, particularly compounds that inhibit viral integrase.

SUMMARY OF THE INVENTION

Purified antiviral compounds, pharmaceutical formulations containing the compounds, and methods of use of the compounds are provided. The compositions of the invention are isolated antiviral components from plant extracts that find use in the treatment of viral infections. In particular, it is believed that the compounds of the invention inhibit the activity of viral integrase. The purified compounds are represented by Formula (I):



wherein:

each R is independently H or an alkyl group, such as a C1-C4 alkyl; and

R_1 is H, alkyl, substituted alkyl, aryl or substituted aryl, and pharmaceutically acceptable salts thereof.

Preferably, each R is selected from the group consisting of H and methyl and R_1 is an alkyl group substituted with a substituted phenyl group and a carboxyl group. Preferred substituents on the phenyl ring include one or more hydroxyl groups. Preferably, the purified compounds have a purity of at least about 90%, more preferably at least about 95%, and most preferably at least about 99%.

Methods for the isolation of the antiviral compounds are also provided. The isolation method comprises providing plant material, such as *Salvia miltiorrhiza*, and

extracting an alcohol-soluble fraction therefrom. The roots of the *Salvia* plant are particularly preferred. A group of compounds are precipitated from the alcohol-soluble fraction and separated into an aqueous layer and an organic layer. Thereafter, chromatography can be used to isolate at least one compound of Formula (I) from the organic layer. For example, high performance liquid chromatography (HPLC) can be used in the isolation step.

Methods for treatment or prevention of viral infection, or the disease that it causes it, using the compounds of Formula (I) are also provided. The method involves administering to a population of cells, either *in vitro* or *in vivo*,. a therapeutically effective amount of a purified compound of Formula (I) or a pharmaceutically acceptable salt thereof. The compound of Formula (I) may be administered alone or as part of a pharmaceutical composition comprising the purified compound, one or more pharmaceutically acceptable carriers and, optionally, one or more additional antiviral agents. In specific embodiments, methods are provided to treat and/or prevent HIV-1 infection and to treat and/or prevent AIDS by inhibiting the integrase activity of HIV-1. The compositions of the invention find use in inhibiting viral replication in a mammal, particularly a human being.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus described the invention in general terms, reference will now be made to the accompanying drawings, wherein:

Figures 1A and 1B show the inhibition of 3′ processing activities of HIV-1 integrase (IN) in the presence of varying concentrations of P.8s and P.8p fractions;

Figures 2A and 2B show the inhibition of 3′ processing activities of HIV-1 integrase (IN) in the presence of different concentrations of butanol fraction (Bu.M_5);

Figure 3 shows the HPLC chromatogram of the butanol fraction;

Figures 4A and 4B show the HPLC chromatograms of separately pooled $M_5$22 and $M_5$32 peaks from Figure 3 reapplied to the column;

Figures 5A and 5B show the effect of $M_5$22 compound on the inhibition of catalytic activities of HIV-1 integrase (IN) in the presence of different concentrations;

Figures 6A and 6B show the effect of M_532 compound on the inhibition of catalytic activities of HIV-1 integrase (IN) in the presence of different concentrations;

Figures 7A and 7B show the effect of M_522 and M_532 on HIV-1 replication and cell viability; and

Figure 8 shows the inhibition of HIV-1 replication on four different virus strains by using the M_522 compound in H9 cells.

DETAILED DESCRIPTION OF THE INVENTION

The present invention now will be described more fully hereinafter with reference to the accompanying drawings, in which preferred embodiments of the invention are shown. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

The terms "alkyl," "alkene," and "alkoxy" include straight chain and branched alkyl, alkene, and alkoxy, respectively. The term "lower alkyl" refers to C1-C4 alkyl. The term "alkoxy" refers to oxygen substituted alkyl, for example, of the formulas –OR or –ROR^1, wherein R and R^1 are each independently selected alkyl. The terms "substituted alkyl" and "substituted alkene" refer to alkyl and alkene, respectively, substituted with one or more non-interfering substituents, such as but not limited to, C3-C6 cycloalkyl, e.g., cyclopropyl, cyclobutyl, and the like; acetylene; cyano; alkoxy, e.g., methoxy, ethoxy, and the like; lower alkanoyloxy, e.g., acetoxy; hydroxy; carboxyl; amino; lower alkylamino, e.g., methylamino; ketone; halo, e.g. chloro or bromo; phenyl; substituted phenyl, and the like. The term "halogen" includes fluorine, chlorine, iodine and bromine.

"Aryl" means one or more aromatic rings, each of 5 or 6 carbon atoms. Multiple aryl rings may be fused, as in naphthyl or unfused, as in biphenyl. Aryl rings may also be fused or unfused with one or more cyclic hydrocarbon, heteroaryl, or heterocyclic rings.

"Substituted aryl" is aryl having one or more non-interfering groups as

substituents.

"Non-interfering substituents" are those groups that yield stable compounds. Suitable non-interfering substituents or radicals include, but are not limited to, halo, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_1-C_{10} alkoxy, C_7-C_{12} aralkyl, C_7-C_{12} alkaryl, C_3-C_{10} cycloalkyl, C_3-C_{10} cycloalkenyl, phenyl, substituted phenyl, toluoyl, xylenyl, biphenyl, C_2-C_{12} alkoxyalkyl, C_7-C_{12} alkoxyaryl, C_7-C_{12} aryloxyalkyl, C_6-C_{12} oxyaryl, C_1-C_6 alkylsulfinyl, C_1-C_{10} alkylsulfonyl, $-(CH_2)_m-O-(C_1-C_{10}$ alkyl) wherein m is from 1 to 8, aryl, substituted aryl, substituted alkoxy, fluoroalkyl, heterocyclic radical, substituted heterocyclic radical, nitroalkyl, $-NO_2$, -CN, -NRC(O)-(C_1-C_{10} alkyl), -C(O)-(C_1-C_{10} alkyl), C_2-C_{10} thioalkyl, -C(O)O-(C_1-C_{10} alkyl), -OH, $-SO_2$, =S, -COOH, $-NR_2$, carbonyl, -C(O)-(C_1-C_{10} alkyl)-CF_3, -C(O)-CF_3, -C(O)NR_2, -(C_1-C_{10} alkyl)-S-(C_6-C_{12} aryl), -C(O)-(C_6-C_{12} aryl), $-(CH_2)_m-O-(CH_2)_m-O-(C_1-C_{10}$ alkyl) wherein each m is from 1 to 8, -C(O)NR_2, -C(S)NR_2, -SO_2NR_2, -NRC(O)NR_2, -NRC(S)NR_2, salts thereof, and the like. Each R as used herein is H, alkyl or substituted alkyl, aryl or substituted aryl, aralkyl, or alkaryl.

The present invention is drawn to compounds and compositions which find use as antiviral agents. The present invention is also drawn to the method of isolating these compounds from plant extracts and using them to treat a variety of viral infections.

Due to the lack of toxicity and the low IC_{50} values for inhibition of HIV-1 replication and HIV-1 integrase activity exhibited by the compounds of Formula (I) (See Examples), it is believed that purified compounds of Formula (I) are potent antiviral agents and can likely be useful as therapeutic drugs for AIDS, as well as other viruses.

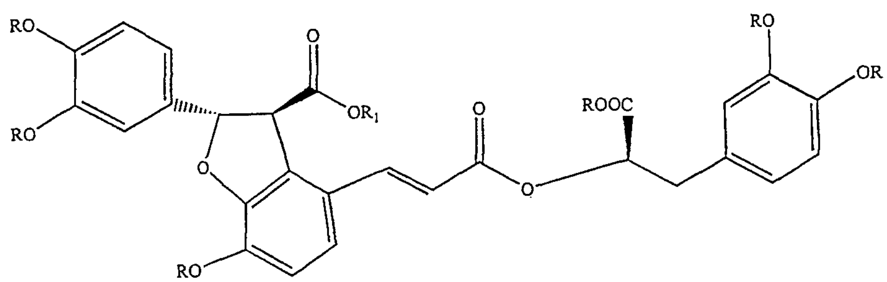
The compounds and compositions of the invention have anti-viral activities. The phrase "antiviral activity" is used herein to mean the effective inhibition of the activity of a virus, including, but not limited to, its propagation or replication. Generally, virus replication includes cell entry, integration into the genome, transcription of the viral genome, translation of viral proteins, post-translational modifications, assembly of virion components, and release. Thus, the compositions of

the invention effectively inhibit at least one aspect of the replication cycle. Assays can be performed to identify the mechanism by which the composition functions to inhibit viral activity. Such assays are well known in the art. See, for example, Lee et al. (1994) *Analytical Biochemistry* 220, 377-383; Lee et al. (1995) *Analytical Biochemistry* 227, 295-301; U.S. Patent application No. 08/365,473; Lee et al. (1995) *Biochemistry* 34, 10205-10214; Lee et al. (1995) *Biochemistry* 34, 10215-10223; Lee and Han (1996) *Biochemistry* 35, 3837-3844.

The present invention encompasses purifying or isolating these antiviral compounds from plants. A purified or isolated compound is substantially free of other compounds. By "substantially free" is intended a purity of at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 99.5%. NMR profiles of samples of $M_5$22 and $M_5$32 compounds have confirmed a purity level of at least about 99 to 99.5%. By "purity" is intended the percentage of the dry weight of the compound of interest divided by the dry weight of the purified fraction containing the compound of interest.

The present invention also encompasses using compositions comprising such isolated compounds for the treatment of a variety of viral infections. By "treatment" is intended the either the reduction of the total number of viral particles, the retardation of viral propagation, or the relief or prevention of symptoms caused by viral infection in a patient following administration of these compositions. Assays can be performed to determine the inhibitory effect of a composition on viral activities, which include, but are not limited to, immunoassays detecting viral antigens, such as viral surface antigens or core antigens. Such assays are well known in the art.

The compositions of the invention comprise a purified compound of Formula (I):

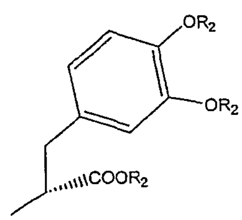


wherein:

each R is independently H or an alkyl group, such as a C1-C4 alkyl; and

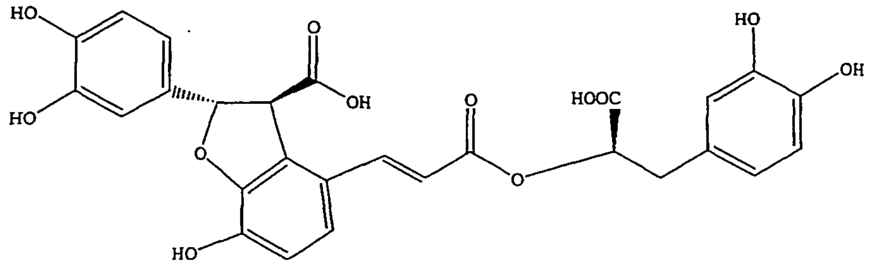
R_1 is H, alkyl, substituted alkyl, aryl or substituted aryl.

Preferred R substituents include H or methyl. Preferred R_1 substituents include alkyl groups substituted with a carboxyl group and a substituted phenyl ring, such as a phenyl ring substituted with one or more hydroxyl groups. In one preferred embodiment, R_1 and each R are H. In another embodiment, each R is H and R_1 is



wherein each R_2 is independently H or alkyl, such as lower alkyl.

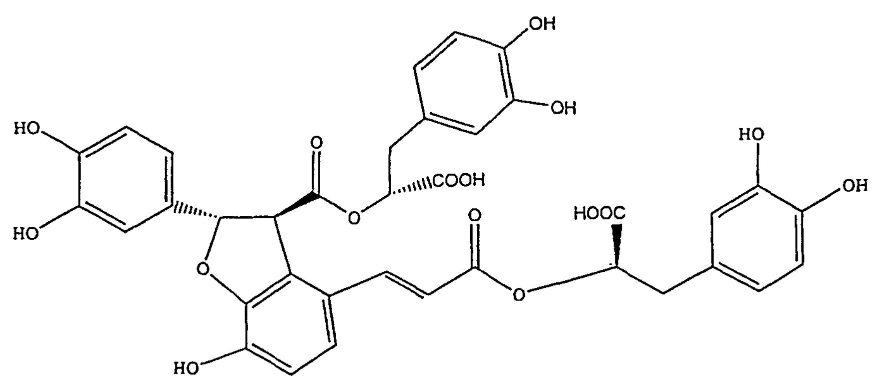
One preferred embodiment, known as lithospermic acid (also referred to as M_522 herein), is shown below. The molecular weight of lithospermic acid is 538.



Lithospermic Acid (M_522)

(4-{2-[l-carboxy-2-(3,4-dihydroxy-phenyl)-ethoxycarbonyl]-vinyl}-2-(3,4-dihydroxy-phenyl)-7-hydroxy-2,3-dihydro-benzofuran-3-carboxylic acid)

Another preferred embodiment, known as lithospermic acid B (also referred to as M_532 herein), is shown below. The molecular weight of lithospermic acid B is 718.



Lithospermic Acid B (M_532)

(4-{2-[l-carboxy-2-(3,4-dihydroxy-phenyl)-ethoxycarbonyl]-vinyl}-2-(3,4-dihydroxy-phenyl)-7-hydroxy-2,3-dihydro-benzofuran-3-carboxylic acid 1-carboxy-2-(3,4-dihydroxy-phenyl)-ethyl ester)

Viral integration is an attractive target because there is no apparent human protein counterpart, the steps involved in proviral integration are similar for all retroviruses, and the structural and functional properties among all types or classes of retroviral integrases are similar. See Khan et al. (1991) *Nucleic Acids Research* 19, 851-860; Thomas and Brady (1997) *Trends in Biotechnology* 15, 167-172. These publications are herein incorporated by reference. Thus, an inhibitor against integrase can be used as an antiviral therapy for a broad range of virus infections including HIV, SIV (simian immunodeficiency virus); MuLV (mouse leukemia virus); and the like.

Assays can be performed to test the inhibitory effect of a composition on the integration of the viral DNA into the host genome. For example, assays to test the ability of the composition to inhibit viral integrase activity are known in the art. Integrase specifically recognizes both ends of the viral DNA and removes two nucleotides from the 3′ ends. The processed viral DNA and integrase then migrate to the nucleus where a viral integrase covalently links the viral genome to host chromosomal DNA, known as strand transfer, forming the provirus. Thus, inhibition of viral integrase results in inhibition of the integration of the viral DNA into the genome of the infected cells and the replication of the viral DNA. Thus, the level of integrase activity can be assayed by measuring the degree to which the ends of viral DNA or fragments containing viral DNA sequences are processed. It is recognized that the sequence of the DNA fragment used in the assay will vary according to the recognition specificity of the particular viral integrase that is being assayed. See Chow (1997) *Methods* 12, 306-317; Kulkosky et al. (1995) *Virology* 206, 448-456; Katzman and Katz (1999) *Advances in Virus Research* 52, 371-395. These publications are herein incorporated by reference. A compound that inhibits viral integrase activity can reduce the level of integrase activity by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99%.

The compositions of the invention can be used alone or in combination with other antiviral agents that inhibit the same or different aspects of the viral replication cycle (i.e., integration, viral entry, proviral transcription, viral replication, or viral assembly). Such agents include, but are not limited to, zidovudine (AZT), didanosine

(ddI), stavudine (d4T), zalcitabine (ddC), amantadine interferon, ribavirin, rimantadine, and NDGA derivatives (See Gnabre, *et al.*, 1995, *Proc. Natl. Acad. Sci.*, USA 92, 11239-11243; Hwu, et al., 1998, *J. Med. Chem.*, 41, 2994-3000; Chen, et al., 1998, *J. Med. Chem.*, 41, 3001-3007; U.S. Patent No. 6, 214,874 B).

The methods and compositions of the invention are useful against a wide array of viruses. For example, both simple and complex retroviruses are encompassed by the present invention. Members of those taxonomic divisions are set forth in Table 1.

Table 1: Major taxonomic divisions among retroviruses

Category	Subgroup	Prototype	Other examples
Simple retroviruses	C-type retroviruses Group A	RSV	ALV, ASV
	C-type retroviruses Group B	MLV	FeLV,MSV,SNV, REV,SSV
	B-type retroviruses	MMTV	
	D-type retroviruses	MPMV	SRV-1
Complex retroviruses	Lentiviruses	HIV-1	HIV-2,SIV,visna virus,FIV,EIAV
	T-cell leukemia viruses	HTLV-1	TLV-II,STLV, BLV
	Spumaviruses	HSRV	SFV,BFV

Abbreviations: RSV, Rous sarcoma virus; ALV, avian leukemia virus; ASV, avian sarcoma virus; FeLV, feline leukemia virus; MSV, murine sarcoma virus; SNV, spleen necrosis virus; REV, reticuloendotheliosis virus; SSV, simian sarcoma virus; MMTV, mouse mammary tumor virus; MPMV, Mason-Pfizer monkey virus; SRV-1, simian retrovirus type 1; STLV, simian T-cell leukemia virus; BFV, bovine foamy virus

The methods and compositions of the present invention are also useful in the treatment of diseases and/or clinical symptoms resulting from a viral infection. Such viral infections include, but are not limited to, infections caused by the complex group of retroviruses including all lentiviruses, spumaviruses as well as HTLV-1 and related viruses, which are responsible for diseases such as acquired immunodeficiency syndrome (AIDS) and T-cell leukemias (the human T lymphotrophic virus I, HTLV-I).

The antiviral compositions and compounds of the invention can be purified or partially purified from plants and plant extracts. Of particular interest are plants from the genus *Salvia*, such as *S. miltiorrhiza*, *S. officinalis*, *S. splendens*, *S. lyrata*, *urticifolia*, *S. farinace*, particularly *Salvia miltiorrhiza*. Of particular interest are extracts obtained from plant roots.

Methods are readily available for the partial purification or complete purification of the antiviral compound of the invention. Such methods include, for example, centrifugation, dialysis, solvent extraction (using solvent systems including methanol, dichloromethane, propanol, ethanol, butanol, etc.), precipitation, column separations, chromatography (liquid, anion exchange, cation exchange, thin layer, affinity, hydrophobic interaction, gel filtration, reverse phase, high performance liquid, etc.), mass spectrometry, and the like. Such methods can be used in any sequence or combinations. Generally, the antiviral compounds of the invention can be separated based on chemical and physical properties, such as solvent solubility, molecular size, charge, polarity, and hydrophobicity. For example, after an initial purification using dialysis, extraction and chromatography, tandem mass spectrometry (MS/MS) can be performed on the sample extract. MS/MS is utilized when mixtures contain components of the same molecular weight. Likewise, LS/MS/MS is a powerful tool for characterizing samples with large numbers of components.

Methods for such procedures are readily available in the prior art. See, for example, *Perry's Chemical Engineers' Handbook*, Sixth Ed. (Robert H. Perry and Don Green (eds.)) McGraw-Hill, Inc. (1984); *Practical HPLC methodology and applications* (Brian A. Bidlingmeyer) 1992 Wiley, New York; *A Practical guide to HPLC detection* (edited by Donald Parriott) 1993 Academic Press, Inc., San Diego, CA; *Solvent extraction in analytical chemistry* (George H. Morrison and Henry Freiser) 1957 Wiley New York; *Solvent extraction in biotechnology: recovery of primary and secondary metabolites* (Karl Schugerl) 1994 Springer-Verlag Berlin, New York; *Interpretation of mass spectra of organic compounds* (Mynard C. Hamming and Norman G. Foster) 1972 Academic Press, Inc. New York; Youngquist et al. (1995) *J. Am. Chem. Soc.* 117: 3900-3906; Dunayevskiy et al (1995) *Anal.*

Chem. 67: 2906-2915; Brummel et al. (1996) *Anal. Chem.* 68: 237-242; Metzger et al. (1994) *Analytical Biochemistry* 219: 261-277; Brummel et al. (1994) *Science* 264: 399-402; *Methods in Enzymology*, Vol. 182, *Guide to Protein Purification*, edited by Murray P. Deutscher 1990, Academic Press, Inc. San Diego, CA; herein incorporated by reference.

In a preferred method, the compounds of the invention are isolated or purified from plant material, such as *Salvia miltiorrhiza*. The preferred method comprises extracting an alcohol-soluble fraction from the plant material, such a root material. A group of compounds are then precipitated from the alcohol-soluble fraction and separated into an aqueous layer and an organic layer, such as a butanol-soluble layer. Thereafter, chromatography techniques can be used to isolate at least one compound of Formula (I) from the organic layer. For example, high performance liquid chromatography (HPLC) can be used in the isolation step.

At each stage of the purification process, the resulting fractions can be tested for antiviral activity. In this manner, partially purified extracts, extracts containing at least the active compound of Formula (I), a partially purified compound of Formula (I), or an isolated compound of Formula (I) having antiviral activity can be obtained. Antiviral activity can be tested using standard techniques such as the integrase assay set forth in the Experimental Section or others known in the art.

After purification of antiviral compounds by the methods noted above, various methods are available in the art for the determination of the structure of the isolated compounds; for example, mass spectroscopy and nuclear magnetic resonance (NMR), and the like.

The purified antiviral compounds can be administered in various compositions to a population of cells that are infected with the virus. The population of cells can be cultured *in vitro*, or found *in vivo* in a living organism, more particularly a mammal. *In vitro*, the population of cells can be adherent cells or cells in suspension. *In vivo*, the population of cells can be from any organ or combination of organs of the body of the organism.

The antiviral compositions can be used for the treatment of patients infected with a virus or viruses, when the compositions of the present invention has an inhibitory effect on the activity of such virus. All viral infections in patients are potentially treatable by the composition in the present invention.

The antiviral compositions can be formulated according to known methods to prepare pharmaceutically useful compositions, such as by admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation are described, for example, in *Remington's Pharmaceutical Sciences* (16th ed., Osol, A. (ed.), Mack, Easton PA (1980)). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the antiviral compound, either alone, or with a suitable amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved by the use of polymers to complex or absorb the antiviral compositions. The controlled delivery may be exercised by selecting appropriate macro molecules (for example, polyesters, polyamino acids, polyvinyl pyrrolidone, ethylene-vinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate). The rate of drug release may also be controlled by altering the concentration of such macromolecules.

Another possible method for controlling the duration of action comprises incorporating the therapeutic agents into particles of a polymeric substance such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, it is possible to entrap the therapeutic agents in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, by the use of hydroxymethyl cellulose or gelatin-microcapsules or poly(methylmethacrylate) microcapsules, respectively, or in a colloid drug delivery system, for example, liposomes, albumin, microspheres, microemulsions, nanoparticles, nanocapsules, or in macroemulsions. Such teachings are disclosed in *Remington's Pharmaceutical Sciences* (1980).

It is contemplated that the inhibitory compositions of the present invention will be administered to an individual in therapeutically effective amounts. That is, in an amount sufficient to inhibit the replication and/or propagation of the target virus. The effective amount of the inhibitory composition will vary according to the weight, sex, age, and medical history of the individual. Other factors which influence the effective amount may include, but are not limited to, the severity of the patient's condition, the severity of viral infection, the stability of the antiviral compound, the kinetics of interaction between the virus and the antiviral compound, previous exposure to the inhibitory compound, kidney or other disease, etc. An effective amount can reduce the level of virus activity by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, preferably about 95%, 96%, 97%, or 98%, more preferably about 99%. Typically, a therapeutically effective amount will range from about 0.1 mg to about 300 mg per kg of body weight per day.

The pharmaceutically prepared inhibitory compositions of the invention may be provided to a patient by means well known in the art. Such means of introduction include oral means, intranasal means, subcutaneous means, intramuscular means, intravenous means, intraarterial means, or parenteral means.

The antiviral compounds of the present invention may be dissolved in any physiologically tolerated liquid in order to prepare an injectable bolus. It is generally preferable to prepare such a bolus by dissolving the molecule in normal saline.

Thus, the present invention provides a method of treating AIDS in a subject, comprising administering to the subject an effective amount of a compound of the present invention, such as a compound of Formula (I). The administering step can comprise administering an effective amount of the compound in a pharmaceutically acceptable carrier.

For the purposes of AIDS therapy, a compound of Formula (I) is administered to the subject in an amount sufficient to inhibit HIV-1 from further infecting other cells. However, the therapeutically effective dosage of any specific compound will vary somewhat from compound to compound, patient to patient, and will depend upon the condition of the patient and the route of delivery. When administered conjointly

with other pharmaceutically active agents, even less of the compounds of Formula (I) may be therapeutically effective. The compound of Formula (I) may be administered once or several times a day. The duration of the treatment may be once per day for a period of from two to three weeks and may continue for a period of months or even years. The daily dose can be administered either by a single dose in the form of an individual dosage unit or several smaller dosage units or by multiple administration of subdivided dosages at certain intervals.

The compounds of Formulas (I) may be administered per se or in the form of a pharmaceutically acceptable salt. When used in medicine, the salts of the compounds of Formulas (I) should be both pharmacologically and pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare the free active compound or pharmaceutically acceptable salts thereof and are not excluded from the scope of this invention. Such pharmacologically and pharmaceutically acceptable salts can be prepared by reaction of a compound of Formula (I) with an organic or inorganic acid, using standard methods detailed in the literature. Examples of useful salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicyclic, p-toluenesulfonic, tartaric, citric, methanesulphonic, formic, malonic, succinic, naphthalene-2-sulphonic and benzenesulphonic, and the like. Also, pharmaceutically acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium, or calcium salts of the carboxylic acid group.

Thus the present invention also provides pharmaceutical formulations or compositions, both for veterinary and for human medical use, which comprise the a compound of Formula (I) or a pharmaceutically acceptable salt thereof with one or more pharmaceutically acceptable carriers thereof and optionally any other therapeutic ingredients, such as other chemotherapeutic agents for AIDS. The carrier(s) must be pharmaceutically acceptable in the sense of being compatible with the other ingredients of the formulation and not unduly deleterious to the recipient thereof.

The compositions includes those suitable for oral, rectal, topical, nasal, ophthalmic, or parenteral (including intraperitoneal, intravenous, subcutaneous, or

intramuscular injection) administration. The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier that constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier or both, and then, if necessary, shaping the product into desired formulations.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets, lozenges, and the like, each containing a predetermined amount of the active agent as a powder or granules; or a suspension in an aqueous liquor or non-aqueous liquid such as a syrup, an elixir, an emulsion, a draught, and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine, with the active compound being in a free-flowing form such as a powder or granules which is optionally mixed with a binder, disintegrant, lubricant, inert diluent, surface active agent or dispersing agent. Molded tablets comprised with a suitable carrier may be made by molding in a suitable machine.

A syrup may be made by adding the active compound to a concentrated aqueous solution of a sugar, for example sucrose, to which may also be added any accessory ingredient(s). Such accessory ingredients may include flavorings, suitable preservatives, an agent to retard crystallization of the sugar, and an agent to increase the solubility of any other ingredient, such as polyhydric alcohol, for example, glycerol or sorbitol.

Formulations suitable for parental administration conveniently comprise a sterile aqueous preparation of the active compound, which can be isotonic with the blood of the recipient.

Nasal spray formulations comprise purified aqueous solutions of the active agent with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal mucous membranes.

Formulations for rectal administration may be presented as a suppository with a suitable carrier such as cocoa butter, or hydrogenated fats or hydrogenated fatty carboxylic acids.

Ophthalmic formulations are prepared by a similar method to the nasal spray, except that the pH and isotonic factors are preferably adjusted to match that of the eye.

Topical formulations comprise the active compound dissolved or suspended in one or more media such as mineral oil, petroleum, polyhydroxy alcohols or other bases used for topical formulations. The addition of other accessory ingredients as noted above may be desirable.

Further, the present invention provides liposomal formulations of the compounds of Formula (I) and salts thereof. The technology for forming liposomal suspensions is well known in the art. When the compound of Formula (I) or salt thereof is an aqueous-soluble salt, using conventional liposome technology, the same may be incorporated into lipid vesicles. In such an instance, due to the water solubility of the compound or salt, the compound or salt will be substantially entrained within the hydrophilic center or core of the liposomes. The lipid layer employed may be of any conventional composition and may either contain cholesterol or may be cholesterol-free. When the compound or salt of interest is water-insoluble, again employing conventional liposome formation technology, the salt may be substantially entrained within the hydrophobic lipid bilayer that forms the structure of the liposome. In either instance, the liposomes that are produced may be reduced in size, as through the use of standard sonication and homogenization techniques. The liposomal formulations containing the compounds of Formula (I) or salts thereof, may be lyophilized to produce a lyophilizate which may be reconstituted with a pharmaceutically acceptable carrier, such as water, to regenerate a liposomal suspension.

Pharmaceutical formulations are also provided which are suitable for administration as an aerosol, by inhalation. These formulations comprise a solution or suspension of the desired compound of Formula (I) or a salt thereof or a plurality of solid particles of the compound or salt. The desired formulation may be placed in a

small chamber and nebulized. Nebulization may be accomplished by compressed air or by ultrasonic energy to form a plurality of liquid droplets or solid particles comprising the compounds or salts.

In addition to the aforementioned ingredients, the compositions of the invention may further include one or more accessory ingredient(s) selected from the group consisting of diluents, buffers, flavoring agents, binders, disintegrants, surface active agents, thickeners, lubricants, preservatives (including antioxidants) and the like.

Having now generally described this invention, the same will be better understood by reference to certain specific examples which are included herein for purposes of illustration only, and are not intended to be limiting of the invention, unless specified.

EXPERIMENTAL

The plant, *Salvia miltiorrhiza*, (SM), has been recorded in the Chinese traditional medical book "Ben-cao-gan-mu" with the function of "to activate blood and to resolve stagnant." It showed multiple pharmacological activities both *in vitro* and *in vivo*. Its preparations have been used safely to treat cardiovascular and liver diseases for generations. The herb is widely distributed in China with different species in different districts.

In the appended examples, purification and testing of active anti-HIV compounds from crude extracts of *Salvia miltiorrhiza* roots is described. Two potent, non-toxic HIV-1 integrase inhibitors, M_522 and M_532, were isolated using high performance liquid chromatography (HPLC). Both are pure compounds that showed strong anti-HIV activity in infected H9 cells.

The following cells and virus strains were obtained from the AIDS Research and Reference Regent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: H9 cells, virus strains of HIV-1_{IIIB}A17, a variant resistant to RT nonnucleotide inhibitors and HIV-$1_{L10R/M461/L63P/V82T/184V}$, a protease inhibitor resistant virus, as well as viral strains HTLV-III_{mn}, HIV-1_{RTMF} and HTLV-III_B. Virus-infected

H9 cells were cultured in the presence of different concentrations of M_522 and M_532 and viral replication was assayed using the HIV/p24 monoclonal antibody assay.

The HIV-1 integrase assay utilized in testing described below used a DNA substrate consisting of a DNA sequence derived from the U5 end of HIV-1 LTR. It was prepared by annealing oligonucleotide U5V1 (5'-GACCCTTTTAGTCAGTGT GGAAAATC TCTAGCAGT) with its complementary strand U5V2 (3'-CTGGGAAAATCAGTCACACCTTTTAGAGATCGTCA). The U5V1 strand was labeled at the 5'-end with [γ-^{32}P] ATP using T4 polynucleotide kinase as described previously (Kamata et al., 1994). The standard reaction mixture included reaction buffer (40 mM HEPES, pH 7.5, 20 mM $MnCL_2$, 60 mM NaCl, 20 mM DTT and 0.1% Nonidet - P40) and HIV-1 integrase (NIH AIDS Research and Reference Reagent Program). To assay for 3'-end processing, the labeled substrate was incubated with the reaction mixture for 60 minutes at 37°C. An equal volume of stop solution (95% formamide, 30 mM EDTA, 0.1 % xylene cyanol, 0.1 % bromophenol blue) was added to each reaction and the samples were heated to 95°C for 5 minutes to denature the DNA. The samples were then fractionated by electrophorsis on a 15% denaturing polyacrylamide gel. The 3'-end processing activity was monitored by the appearance of a radioactive oligonucleotides product (33nt), shortened by two nucleotides from the original substrate (35nt). The products were visualized by autoradiography and quantitation was carried out by phosphorimaging.

Example 1

Isolation and Testing of Propanol Soluble (P.8s) and Precipitate (P.8p) Fractions from *Salvia miltiorrhiza roots*

Root powder (1.5 kg) of *Salvia miltiorrhiza* (supplied by Jiang Su Danhua Group Co., China) was extracted three times with 4.5 liters of 70% methanol for 24 hours each time with vigorous shaking. The three extracts (70 S fraction) were combined, evaporated to dryness and dissolved in 2.0 liters of 25% NH_40H. The extract solution was then precipitated with four volumes of 1-propanol and the precipitate was washed three times with 5.0 liters of P.8 buffer (Propanol: H_20:

NH_4OH, 80:15:5). Both the propanol soluble (P.8s) and precipitate (P.8p) fractions were tested for anti-HIV integrase activity.

The results of testing are shown in Figures 1A and 1B. The 3'-end processing product catalyzed by HIV-1 integrase (33nt) and the DNA substrate (35nt) are shown. In Figure 1A, different concentrations of supernatant fraction (P.8s) were tested with HIV-1 integrase: lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3-5) integrase with 0.25, 0.5 and 1.0 μg/ml of P.8s fraction; lane 6, 33 nucleotide marker. In Figure 1B, the 3'-end processing activity of HIV-1 integrase was assayed with the precipitate fraction (P.8p): lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3-12) integrase with 0.006, 0.012, 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 μg/ml of P.8p and lane 13, 33 nucleotide marker. The P.8 precipitate (P.8p), which represented approximately 34% of the root material, showed potent anti-HIV integrase activity in the 3' cleavage activity assay with an IC_{50} of 0.1 μg/ml while the P.8 soluble fraction (P.8s), representing 66% of the starting material was relatively less active (IC_{50} = 1 μg/ml).

To evaluate the potential clinical value of the P.8p fraction, intraperitoneal acute and subacute toxicity tests in mice were conducted for seven days, where P.8p was given once each day for the acute test and twice each day for the subacute test. The toxicity tests for the P.8p fraction were conducted in kunming mice (body weight, 20±1 gm) over a period of seven days. For the acute toxicity test, groups of 10 mice were given doses of 0.45, 0.69, 0.98, 1.60 and 2.00 g/kg once each day and for the subacute toxicity test, mice were given doses of 0.1, 0.2, 0.4 g/kg twice each day.

The number of mouse deaths were recorded daily and LD_{50} was calculated on the 7th day (see Table 2 below). The results showed that the LD_{50} of the P.8p fraction was 1.2 g/ kg for the acute toxicity test and 0.18 g/ kg for the subacute toxicity test. The P.8p extract showed cumulative toxicity in mice when given intraperitoneally in multiple doses. Thus, although the P.8p fraction contained highly active HIV integrase inhibitors, it also contained materials toxic to animals that should be removed.

Table 2 – Acute and Subacute Toxicity Tests of the P.8p Fraction in Mice

Toxicity Test	Administration Route	Dosage (g/kg)	No. mice	Mortality (7^{th} day)	LD_{50} (g/kg) (7^{th} day)
Acute *	IP	2.00	10	10/10	1.20
		1.60	10	8/10	
		0.98	10	4/10	
		0.69	10	0/10	
		0.45	10	0/10	
Subacute**	IP	0.40	10	10/10	0.18
		0.20	10	6/10	
		0.10	10	0/10	

* P.8p was given once each day
** P.8p was given twice each day

Example 2

Isolation and Testing of Butanol Fraction ($Bu.M_5$) from P.8p Fraction

To separate these toxic materials from the antiviral components, a second purification step was employed. For further purification of the P.8p fraction, 50 grams were dissolved in 200 ml of NAA buffer (7 % acetonitrile, O.1M of ammonium acetate; 0.5 % acetic acid) and the cleared NAA fraction was next mixed with 200 ml of 1-butanol and shaken vigorously. The mixture was partitioned into an NAA layer and a butanol layer, which were collected separately. The butanol ($Bu.M_5$) fraction was dried, redissolved in 5% methanol, cleared by centrifugation and then dried again.

The anti-HIV integrase activity of $Bu.M_5$ was tested. The 3'-end processing product catalyzed by HIV-1 integrase (33nt) and the DNA substrate (35nt) are shown in Figures 2A and 2B. In Figure 2A, different concentrations of the $Bu.M_5$ fraction were tested with HIV-1 integrase: lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3-9) integrase with 0.08, 0.16, 0.3, 0.6, 1.25, 2.5 and 5.0 μg/ml of $Bu.M_5$. In Figure 2B, the percent inhibition of HIV-1 integrase activity with

increasing concentrations of Bu.M_5 fraction is illustrated. The IC_{50} of this fraction is 0.6 μg/ml.

Thus, anti-HIV integrase activity was evident (yield < 1 %) in the combined butanol extracts (Bu.M_5).

The toxicity of the Bu.M_5 fraction was tested in C57bl/6 mice by tail vein injections of the Bu.M_5 fraction (2mg/ml in 0.9% NaCl) over a period of seven days at a daily dose of 10 mg/kg, 20 mg/kg and 30 mg/kg, using two mice per dosage group. The Bu.M_5 fraction showed no toxicity in C57bl/6 mice, with no weight loss observed after tail vein injections of 10mg-30mg/kg each day for seven days. Thus, it is believed that the butanol-soluble fraction of the P.8p fraction would also be useful as an antiviral agent.

Example 3

Purification, Identification and Testing of HIV-integrase Active Compounds from Bu.M_5 Fraction

A. Isolation of Active Compounds

High performance liquid chromatography (HPLC) was used to further purify the Bu.M_5 fraction and to isolate pure anti-HIV compounds from *S. miltiorrhiza* roots. A limited amount (100 mg) of butanol fraction (Bu.M_5) was used for each run. HPLC was performed on Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode array detector. The extract solution was separated and analyzed by using a 250 x 10 mm. preparative C18 (8μm) column with the mobile phase consisting of 5% methanol. The flow rate was 1.0 ml/min and the elution was monitored at a wavelength of 254 nm to facilitate the detection of the different compounds. Figure 3 is a HPLC chromatogram of the Bu.M_5 fraction. Many peaks were resolved at different retention times. The activity of each one was tested against HIV-1 integrase activity to identify the active compounds.

Two major peaks with retention times of 22.4 minutes and 31.4 minutes ($M_5$22 and $M_5$32) were well separated from a large, exceedingly complex mixture of unresolved compounds. These two major peaks were separately pooled and then

reapplied to the column. Figures 4A and 4B are the HPLC chromatograms of separately pooled M_522 and M_532 peaks from Fig. 3 that were reapplied to the column. Figure 4A shows a single and major peak (M_522) was eluted at 22.4 retention time. Figure 4B shows one major peak (M_532) that eluted at about 31.4 retention time. Both M_522 and M_532 were found to be 99%+ pure compounds by NMR analysis.

Approximately 500 runs were made to isolate M_522 and M_532. The yield for M_522 and M_532 from the Bu.M_5 fraction was approximately 13% and 26%, respectively. An overall yield from the initial *Salvia miltiorrhiza* roots of 0.018 % and 0.038 % for M_522 and M_532 was obtained. A brief summary of the purification and the HPLC profiles for compounds M_522 and M_532 are shown in Table 3 below.

Table 3 - A summary of the Purification of HIV-1 Inhibitors from *S. miltiorrhiza* Roots

	Fraction	Amount (g)	Yield (%)
	S. miltiorrhiza roots	1500	
Step I	P.8p	516	34.4
Step II	Bu.M_5	2.2	0.15
Step III	HPLC pure compounds:		
	M_522	0.277	0.018
	M_532	0.572	0.038

B. Identification of Isolated Compounds

The structures of M_522 and M_532 were identified by NMR and MS analysis using known structural standards for comparison (Tanaka et al., 1989). M_522 was identified as lithospermic acid: (4-{2-[l-carboxy-2-(3,4-dihydroxy-phenyl)-ethoxycarbonyl]-vinyl}-2-(3,4-dihydroxy-phenyl)-7-hydroxy-2,3-dihydro-benzofuran-3-carboxylic acid). M_532 was identified as lithospermic acid B: (4-{2-[l-carboxy-2-(3,4-dihydroxy-phenyl)-ethoxycarbonyl]-vinyl}-2-(3,4-dihydroxy-phenyl)-7-hydroxy-2,3-dihydro-benzofuran-3-carboxylic acid 1-carboxy-2-(3,4-dihydroxy-phenyl)-ethyl ester).

C. Efficacy Testing of Isolated Compounds

The anti-HIV efficacy of the two isolated compounds was analyzed by their effect on the 3' processing activity assay and their inhibition of HIV in cultured H9 cells. The inhibitory data indicate that the two compounds are highly potent against HIV integrase, with IC_{50} of 0.45 μg/ml, 0.83 μM for $M_5$22 and 0.35 μg/ml, 0.45 μM for $M_5$32. These results are illustrated in Figures 5 and 6.

Figs. 5A and 5B illustrate the effect of $M_5$22 on the inhibition of HIV-1 integrase (IN) activity in the presence of different concentrations. In Figure 5A, the 3'-end processing product catalyzed by HIV-1 integrase (33nt) and the DNA substrate (35nt) are shown: lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3-9) integrase with 0.15, 0.3, 0.6,1.25, 2.5, 5.0, and 10 μg/ml of $M_5$22 and lane 10, 33 nucleotide marker. Figure 5B is a quantitation of assay results by phosphorimager showing a dose-response curve for the inhibition of HIV-1 integrase activity by the $M_5$22 compound. The IC_{50} of $M_5$22 is 0.45 μg/ml, 0.83 μM.

Figs. 6A and 6B illustrate the effect of $M_5$32 on the inhibition of HIV-1 integrase (IN) activity in the presence of different concentrations. In Figure 6A, the 3'- end processing product catalyzed by HIV-1 integrase (33nt) and the DNA substrate (35nt) are shown: lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3-9) integrase with 0.15 0.3, 0.6,1.25, 2.5, 5.0, and 10 μg/ml of $M_5$32 and lane 10, 33 nucleotide marker. Figure 6B is a quantitation of assay results by phosphorimager showing a dose-response curve for the inhibition of HIV-1 integrase activity by the $M_5$32 compound. The IC_{50} of $M_5$32 is 0.35 μg/ml, 0.45 μM.

The inhibitory effect of $M_5$22 and $M_5$32 against HIV replication was further examined using HIV-1_{IIIB}A17, a variant resistant to RT nonnucleotide inhibitors and HIV-$1_{L10R/M461/L63P/V82T/184V}$, a protease inhibitor resistant virus, as testing viruses in the presence of a variety of drug concentrations in infected H9 cells. By using the HIV/p24 monoclonal antibody assay, the IC_{50} for $M_5$22 and $M_5$32 against HIV replication were found to be 1.5μg/ml, 2.7 μM for $M_5$22 and 1 μg/ml, 1.39 μM for $M_5$32.

Figure 7A graphically illustrates the effect of M_522 and M_532 on HIV-1 replication. Figure 7A illustrates the dose-dependent inhibition of HIV-1 replication in H9 cells infected with HIV-1_{IIIB}A17 and HIV-$1_{L10R/M46I/L63P/V82T/I84V}$ viruses in the presence of different drug concentrations of M_522 and M_532. Viral replication was measured using the HIV/p24 monoclonal antibody assay and the IC_{50} values for inhibition by M_522 and M_532 against HIV replication were calculated as 2.7 μM and 1.39 μM respectively.

D. Cytotoxicity of Isolated Compounds

The cytotoxicity of M_522 and M_532 drugs against H9 cells was analyzed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma Chemical Co.) (Uckun et al., 1998). Briefly, exponentially growing H9 cells were seeded onto 96-well plates at a density of $3x10^4$ cells/well and incubated for 24 h at 37°'C prior to drug exposure. On the day of treatment, a series Of M_522 and M_532 concentrations (1.25, 2.5, 5, 10, 20, 40, 80, and 160 μg/ ml) were used to test their cytotoxicity in H9 cells. Quadruplicate wells were used for each treatment. The cells were incubated with M_522 and M_532 for 4 days at 37°C in a humidified 5% CO_2 atmosphere. To each well, 50 μl of MTT (1mg/ml final concentration) was added and the plates were incubated at 37°C for 4 h to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized with DMSO. The absorbance of each well was measured in a microtiter reader at optical density 540 nm. The reaction is specific, no significant amounts of formazan can be detected with dead cells. We found that the cells remained viable even at the highest concentrations (CC_{100}) of M_522 and M_532 tested (160 μg/ml, 297 μM for M_522 and 160 μg/ml, 223 μM for M_532), indicating that both compounds are non-toxic. These results are presented graphically in Figure 7B.

Example 4

$M_5$22 and $M_5$32 Inhibit the Replication of Four Different Virus Strains in Human H9 Cells

Inhibitory activity of $M_5$22 and $M_5$32 against the replication of four different HIV-1 strains was tested in this example. Two of the strains were drug-resistant virus isolates (HIV-1_{RTMF} resistant against AZT and HIV-1_{IIIB}A17 variant resistant against inhibition by RT nonnucleotide inhibitors) and the other two were primary isolates (HTLV-III_{MN} and HTLV-III_B).

On the day before infection, H9 cells were subcultured at 1-2 x 10^5 cells/ml and on the day of the infection, cells were pelleted by centrifugation at room temperature for 10 minutes. The pellet was then resuspended with 1 ml of each strain according to the virus titer. The mixtures were incubated at 37 °C in CO_2 incubator for 2 hours. The cells were then washed two times with PBS and then two times with the culture medium (RPMI 1640). The cells were resuspended with fresh medium and plated in 96 well plates. Every 3-4 days after infection, cells were sub-cultured and supernatant was saved to assay for virus production.

The activity of $M_5$22 against the four strains were tested at various drug concentrations of 80, 40, 20, 10, 5, 2.5, 1.25, and 0 μg/ml. After 8 days, the infection of the cells was detected by using HIV-1 p24 antigen assay, which is an enzyme immunoassay (EIA, or enzyme-linked immunosorbent assay) developed for detection and quantitation of the HIV-1 p24 core protein. The percentage of HIV-1 inhibition achieved by the different concentrations of $M_5$22 are shown in Table 4 below. The IC_{50} of $M_5$22 for HIV-1_{RTMF}, HIV-1 III_B A17, HTLV-III_B and HTLV-III_{MN} were 2.7 μg/ml, 1.5 μg/ml, 11 μg/ml, and 9 μg/ml, respectively. These results are illustrated graphically in Figure 8.

Table 4

Inhibition of Four Strains HIV-1 Replication by M_522 Drug

% Inhibition

	HIV-1 $_{RTMF}$	HTLV-III $_{MN}$	HIV-III$_B$A17 VARIANT	HTLV-III$_B$
00. μg/ml	0.0	0.0	0.0	0.0
1.25 μg/ml	26.2	7.7	51.2	31.4
2.5 μg/ml	47.7	21.8	53.6	42.1
5.0 μg/ml	78.6	30.0	76.8	45.9
10.0 μg/ml	83.4	51.9	85.0	47.3
20.0 μg/ml	95.5	72.1	90.2	62.0
40.0 μg/ml	98.2	77.3	100.0	82.4
*80.0 μg/ml	100.0	88.4	100.0	94.5

* The concentration of 80.0 μg/ml is equal to 148.5 μM

The same type of test was conducted to examine the effect of M_532 against the same four strains of HIV-1. The percentage of HIV-1 inhibition achieved by the different concentrations of M_532 are shown in Table 5 below. The IC_{50} of M_532 for HIV-1$_{RTMF}$, HTLV-III$_{MN}$, HIV-1 III$_B$ A17 and HTLV-III$_B$ were 5.6 μg/ml, 5.5 μg/ml, 5.0 μg/ml, and 11 μg/ml, respectively.

Table 5

Inhibition of Four Strains HIV-1 Replication by $M_5$32 Drug

% Inhibition

	HIV-1 $_{RTMF}$	HTLV-III $_{MN}$	HIV-III$_B$A17 VARIANT	HTLV-III$_B$
00. μg/ml	0.0	0.0	0.0	0.0
1.25 μg/ml	20.8	8.3	2.0	4.7
2.5 μg/ml	38.7	45.7	21.8	16.1
5.0 μg/ml	44.2	47.0	50.0	20.1
10.0 μg/ml	73.6	57.2	53.6	34.5
20.0 μg/ml	90.4	87.0	97.5	90.8
40.0 μg/ml	95.0	90.0	97.7	96.2
*80.0 μg/ml	98.3	94.1	98.4	96.8

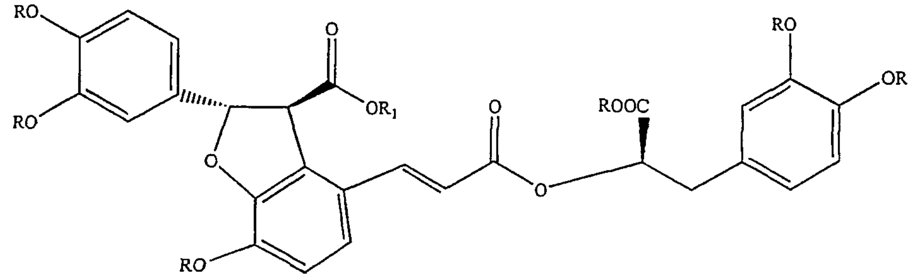
* The concentration of 80.0 μg/ml is equal to 111.5 μM

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Many modifications and other embodiments of the invention will come to mind to one skilled in the art to which this invention pertains having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the invention is not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

THAT WHICH IS CLAIMED:

1. A purified compound having the Formula (I):



wherein each R is independently H or an alkyl group and R_1 is H, alkyl, substituted alkyl, aryl or substituted aryl, or a pharmaceutically acceptable salt thereof.

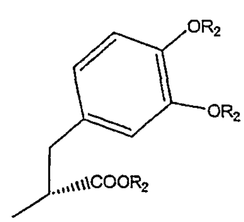
2. The compound of Claim 1, wherein each R is H.

3. The compound of Claim 1, wherein said purified compound has a purity of at least about 90%.

4. The compound of Claim 1, wherein said purified compound has a purity of at least about 99%.

5. The compound of Claim 1, wherein R_1 is substituted alkyl.

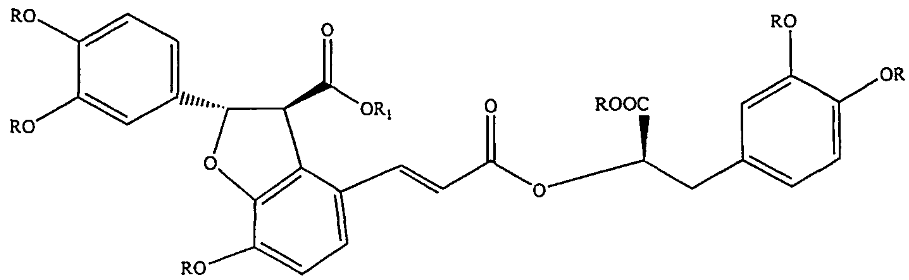
6. The compound of Claim 1, wherein R_1 is:



wherein each R_2 is independently H or alkyl.

7. The compound of Claim 6, wherein each R_2 is H.

8. A pharmaceutical composition, comprising a purified compound having the Formula (I):



wherein each R is independently H or an alkyl group and R_1 is H, alkyl, substituted alkyl,

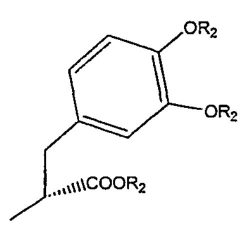
aryl or substituted aryl, or a pharmaceutically acceptable salt thereof; and

a pharmaceutically acceptable carrier.

9. The pharmaceutical composition of Claim 8, wherein each R is H.

10. The pharmaceutical composition of Claim 8, wherein R_1 is substituted alkyl.

11. The pharmaceutical composition of Claim 8, wherein R_1 is:



wherein each R_2 is independently H or alkyl.

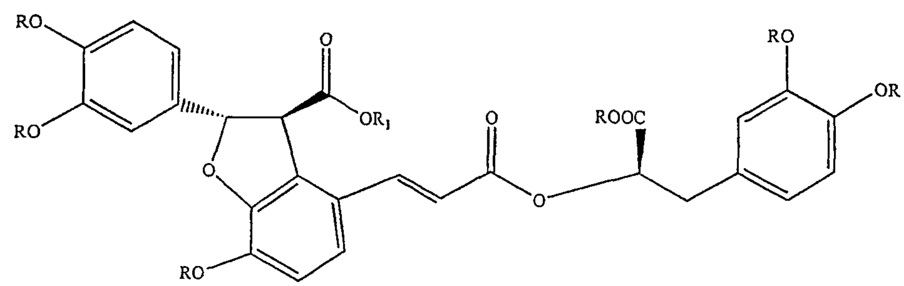
12. The pharmaceutical composition of Claim 11, wherein each R_2 is H.

13. The pharmaceutical composition of Claim 8, wherein said purified compound has a purity of at least about 90%.

14. The pharmaceutical composition of Claim 8, wherein said purified compound has a purity of at least about 99%.

15. The pharmaceutical composition of Claim 8, further comprising at least one additional antiviral agent.

16. A method of treating a virus in a population of cells infected with a virus, comprising administering to the population of cells a therapeutically effective amount of a purified compound of Formula (I):

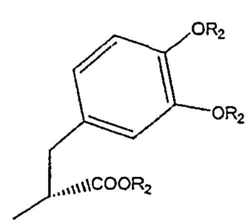


wherein each R is independently H or an alkyl group and R_1 is H, alkyl, substituted alkyl, aryl or substituted aryl, or a pharmaceutically acceptable salt thereof.

17. The method of Claim 16, wherein each R and R_1 are H.

18. The method of Claim 16, wherein R_1 is substituted alkyl.

19. The method of Claim 16, wherein R_1 is:



wherein each R_2 is independently H or alkyl.

20. The method of Claim 19, wherein each R_2 is H.

21. The method of Claim 16, wherein the virus is a retrovirus.

22. The method of Claim 16, wherein the virus is HIV-1.

23. The method of Claim 22, wherein the strain of the HIV-1 virus is selected from the group consisting of HIV-1_{RTMF}, HIV-1 III_B A17 variant, HTLV-III_{MN}, HTLV-III_B, and HIV-$1_{L10R/M461/L63P/V82T/184V}$.

24. The method of claim 16, wherein the purified compound reduces the activity of a viral integrase.

25. The method of Claim 16, wherein the purified compound is administered at a concentration of at least about 0.1 μg/ml.

26. The method of Claim 16, wherein the purified compound is purified from a plant extract.

27. The method of Claim 26, wherein the plant extract is from the genus *Salvia*.

28. The method of Claim 27, wherein the plant extract is *Salvia miltiorrhiza*.

29. The method of Claim 16, wherein the purified compound is administered in a pharmaceutically acceptable carrier.

30. The method of Claim 16, wherein the population of cells are cultured *in vitro*.

31. The method of Claim 16, wherein the population of cells are *in vivo*.

32. The method of Claim 16, wherein the purified compound is administered in combination with at least one additional antiviral agent.

33. A method of purifying a compound of Formula (I) from a plant, said method comprising:

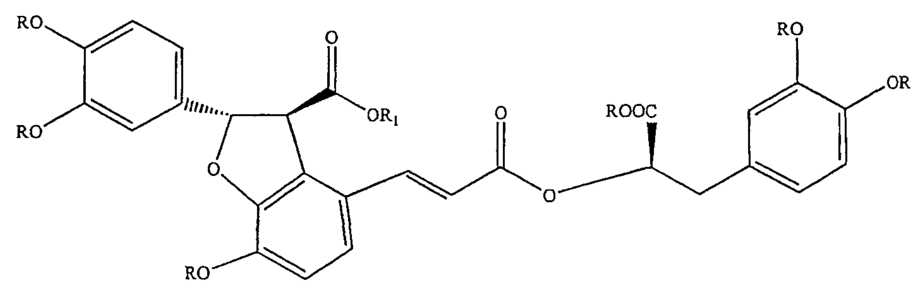
providing plant material from the genus *Salvia*;

extracting an alcohol-soluble fraction from the plant material;

precipitating a group of compounds from the alcohol-soluble fraction;

separating the precipitated compounds into an aqueous layer and an organic layer; and

isolating at least one compound from the organic layer using chromatography, the compound being of Formula (I):

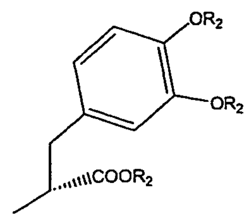


wherein each R is independently H or an alkyl group and R_1 is H, alkyl, substituted alkyl, aryl or substituted aryl.

34. The method of Claim 33, wherein each R and R_1 are H.

35. The method of Claim 33, wherein R_1 is substituted alkyl.

36. The method of Claim 33, wherein R_1 is:



wherein each R_2 is independently H or alkyl.

37. The method of Claim 36, wherein each R_2 is H.

38. The method of Claim 33, wherein the plant material is *Salvia miltiorrhiza.*

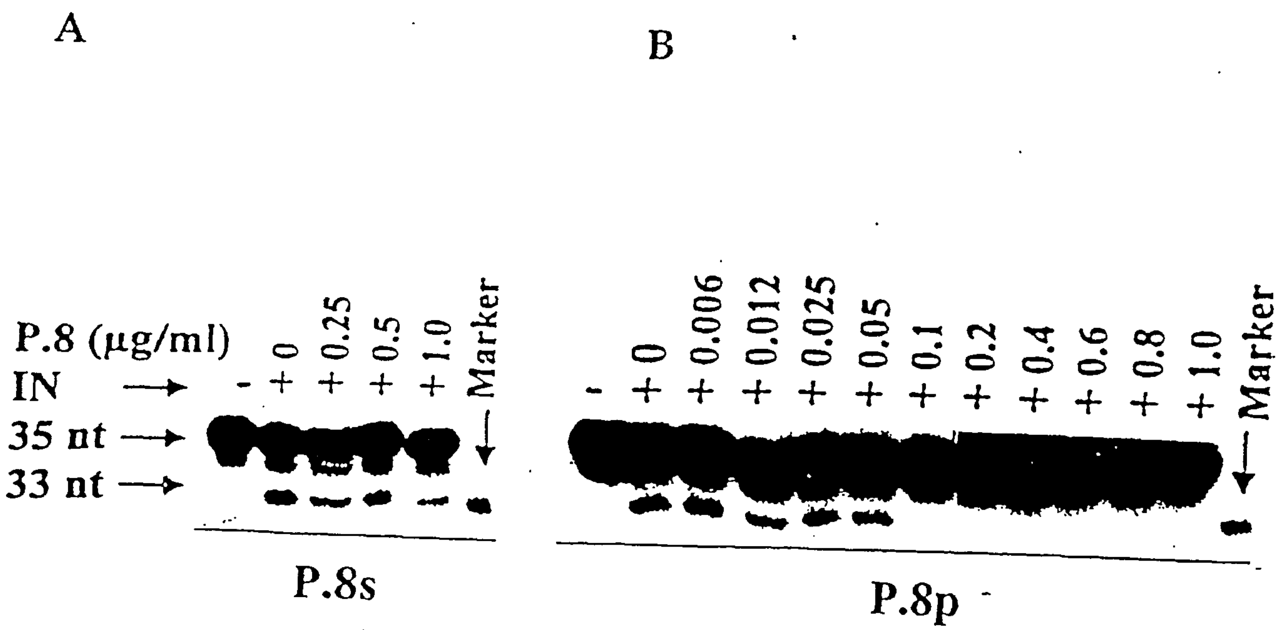
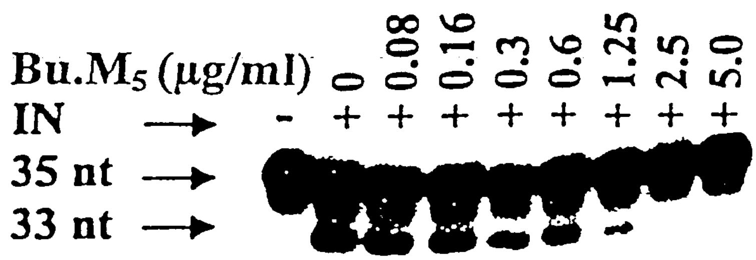

A
B
P.8 (μg/ml)
IN
35 nt
33 nt
- + 0 + 0.25 + 0.5 + 1.0 Marker
P.8s
- + 0 + 0.006 + 0.012 + 0.025 + 0.05 + 0.1 + 0.2 + 0.4 + 0.6 + 0.8 + 1.0 Marker
P.8p

FIGURE 1

A


Bu.M_5 (µg/ml)
0
0.08
0.16
0.3
0.6
1.25
2.5
5.0
IN
- + + + + + + + +
35 nt
33 nt

B

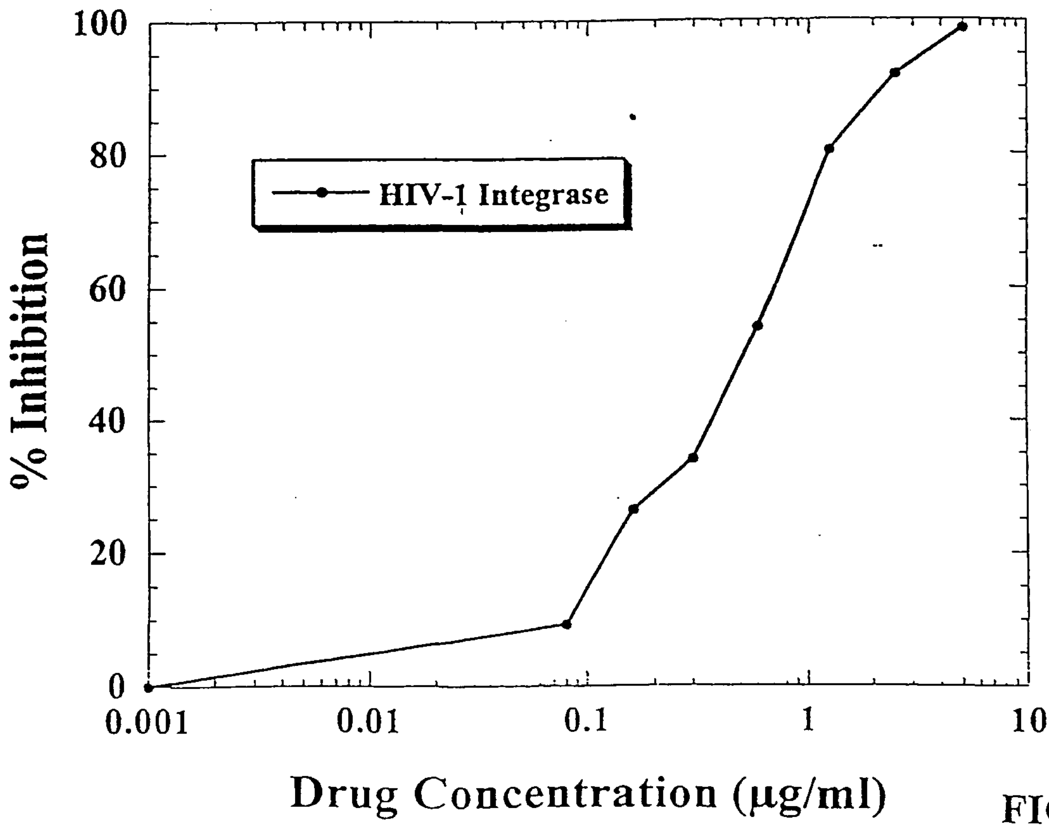

100
80
60
40
20
0
% Inhibition
HIV-1 Integrase
0.001
0.01
0.1
1
10
Drug Concentration (µg/ml)

FIGURE 2

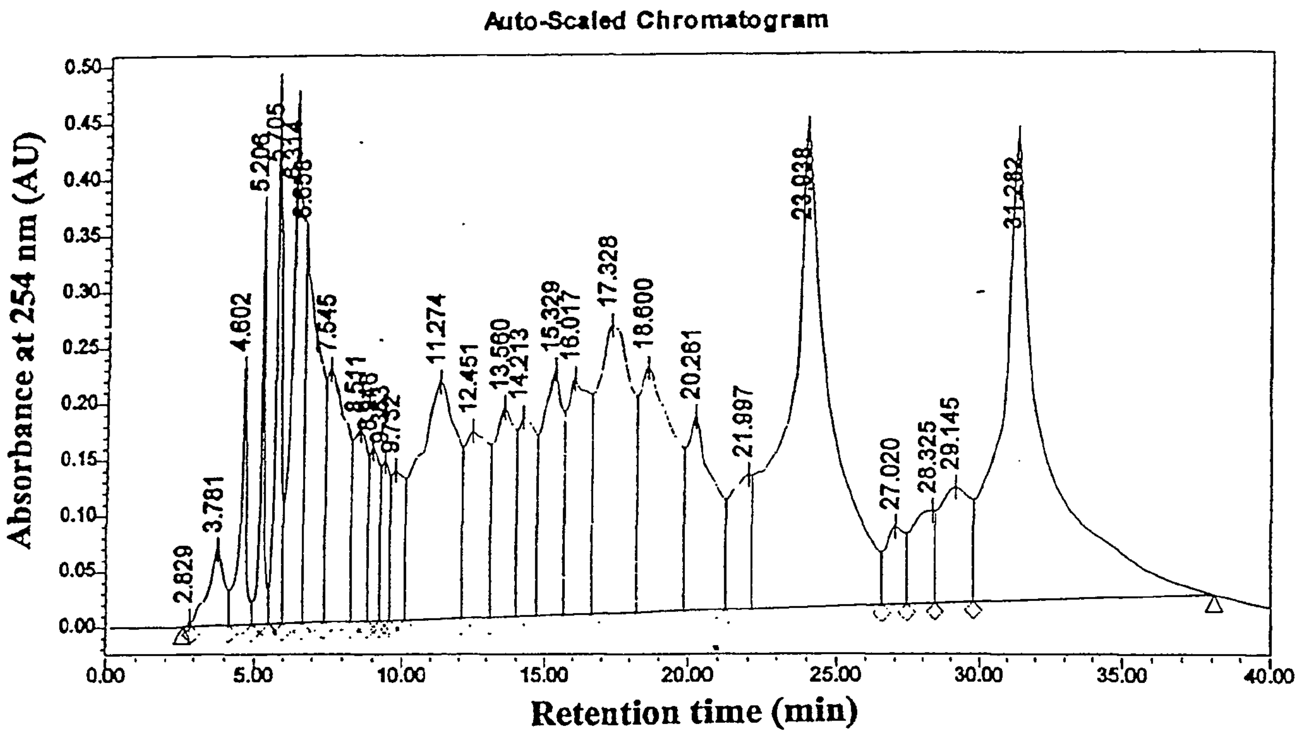

Auto-Scaled Chromatogram
Absorbance at 254 nm (AU)
Retention time (min)
2.829
3.781
4.802
5.206
11.274
12.451
13.580
14.213
15.329
16.017
17.328
18.600
20.261
21.997
23.938
27.020
28.325
29.145
31.282

FIGURE 3

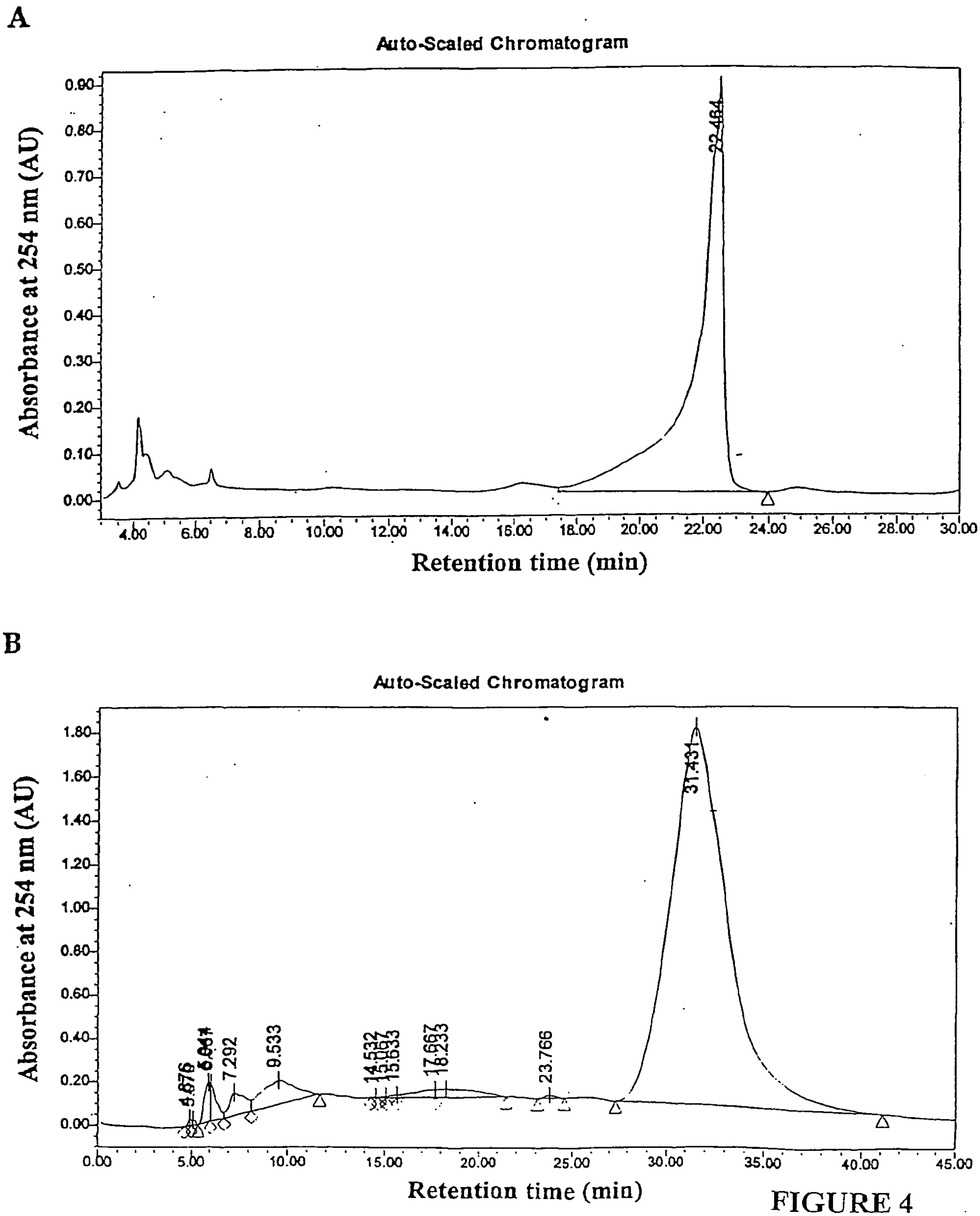
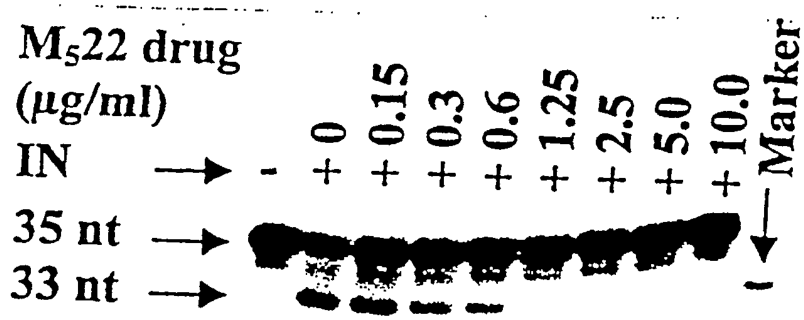

A
Auto-Scaled Chromatogram
Absorbance at 254 nm (AU)
0.00 0.10 0.20 0.30 0.40 0.50 0.60 0.70 0.80 0.90
22.484
4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00 22.00 24.00 26.00 28.00 30.00
Retention time (min)
B
Auto-Scaled Chromatogram
Absorbance at 254 nm (AU)
0.00 0.20 0.40 0.60 0.80 1.00 1.20 1.40 1.60 1.80
31.431
7.292
9.533
23.766
0.00 5.00 10.00 15.00 20.00 25.00 30.00 35.00 40.00 45.00
Retention time (min)

FIGURE 4

A


M5 22 drug
(µg/ml)
IN
0
0.15
0.3
0.6
1.25
2.5
5.0
10.0
Marker
35 nt
33 nt

B

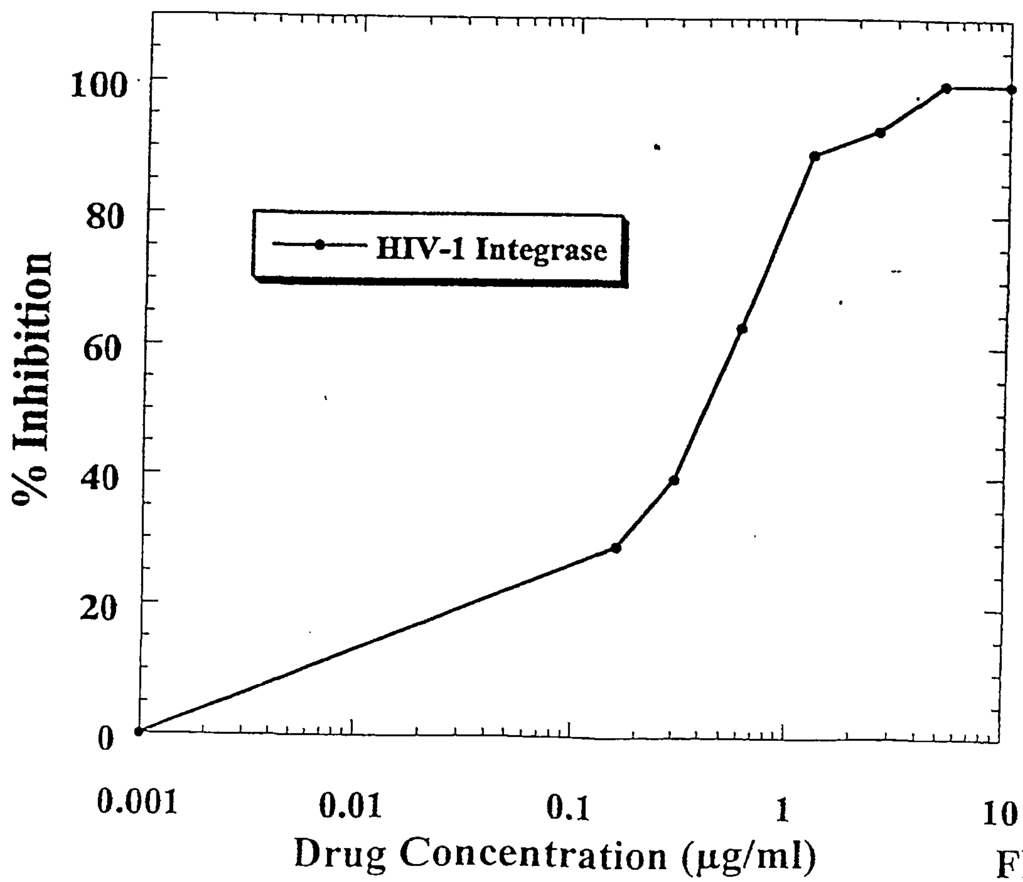
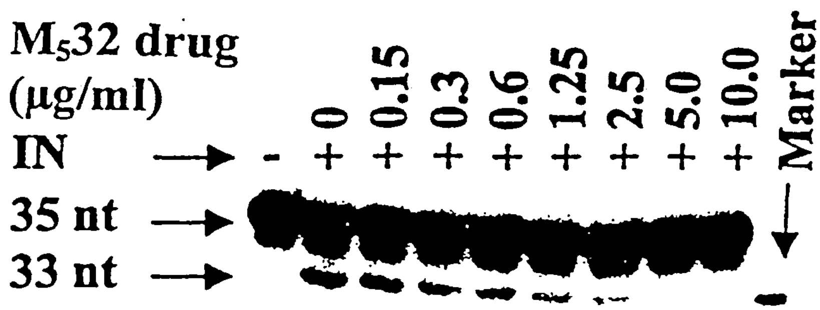

% Inhibition
100
80
60
40
20
0
HIV-1 Integrase
0.001
0.01
0.1
1
10
Drug Concentration (µg/ml)

FIGURE 5

A


M5 32 drug
(µg/ml)
IN
35 nt
33 nt
- + 0 + 0.15 + 0.3 + 0.6 + 1.25 + 2.5 + 5.0 + 10.0
Marker

B

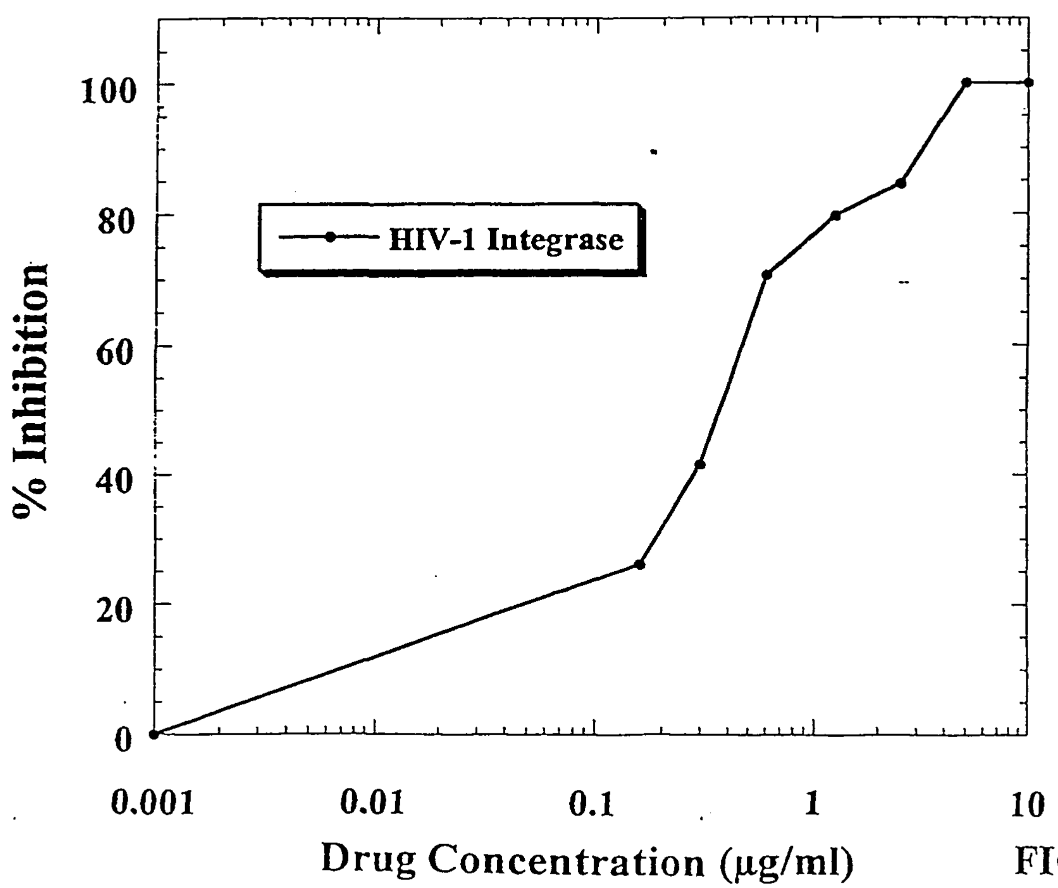

% Inhibition
100
80
60
40
20
0
HIV-1 Integrase
0.001
0.01
0.1
1
10
Drug Concentration (µg/ml)

FIGURE 6

A

B

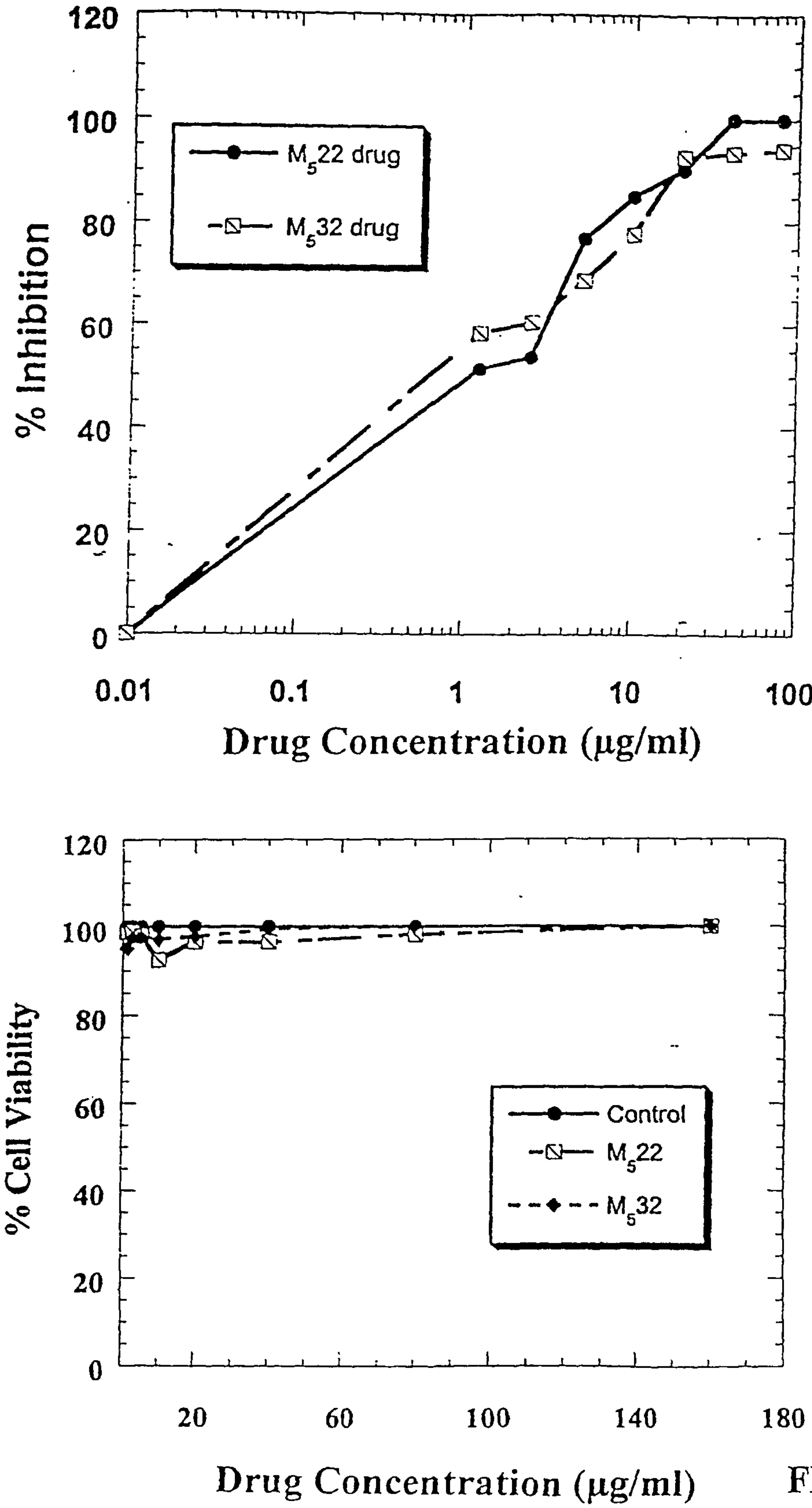

120
100
80
60
40
20
0
% Inhibition
0.01
0.1
1
10
100
Drug Concentration (μg/ml)
M5 22 drug
M5 32 drug
120
100
80
60
40
20
0
% Cell Viability
20
60
100
140
180
Drug Concentration (μg/ml)
Control
M5 22
M5 32

FIGURE 7

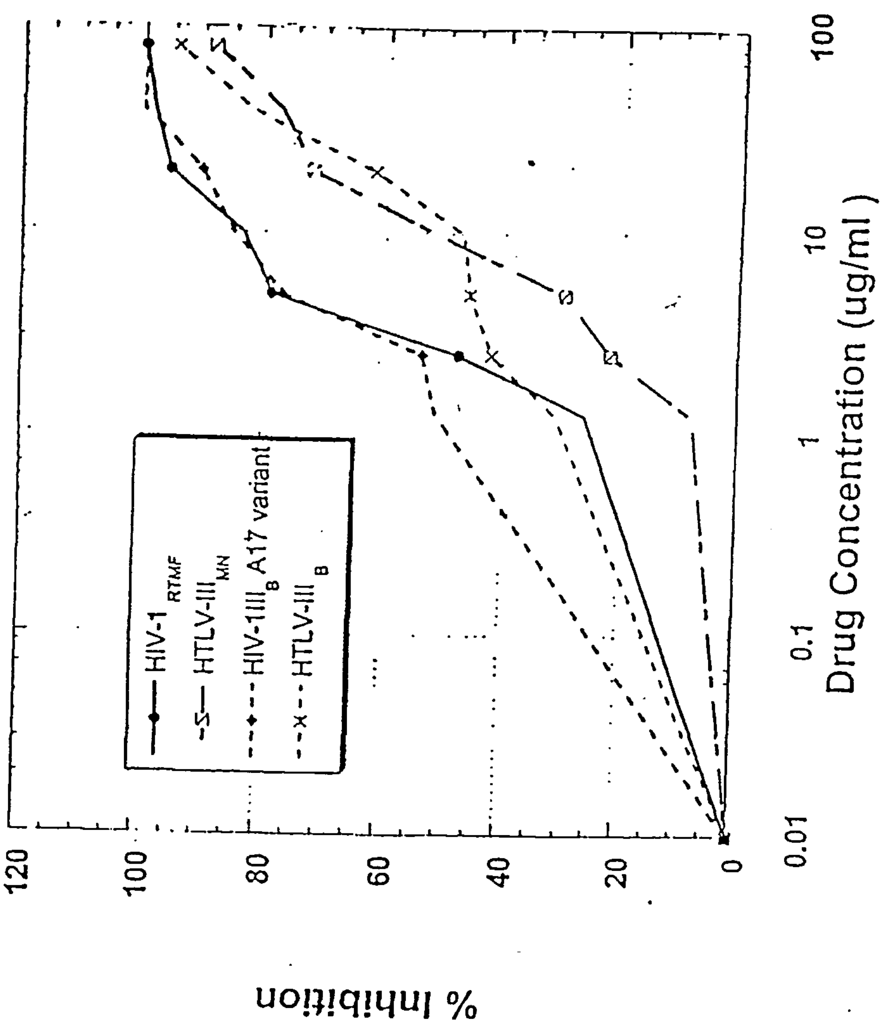

HIV-1 RTMF
HTLV-III MN
HIV-1III B A17 variant
HTLV-III B
% Inhibition
Drug Concentration (ug/ml)
0
20
40
60
80
100
120
0.01
0.1
1
10
100

FIGURE 8

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 April 2002 (04.04.2002)

PCT

(10) International Publication Number
WO 02/026726 A3

(51) International Patent Classification[7]: C07D 307/86, A61K 31/343, A61P 31/18

(21) International Application Number: PCT/US01/29906

(22) International Filing Date: 25 September 2001 (25.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/235,425 26 September 2000 (26.09.2000) US

(71) Applicant (*for all designated States except US*): **THE JOHNS HOPKINS UNIVERSITY** [US/US]; 3400 N. Charles Street, Baltimore, MD 21218 (US).

(72) Inventors; and
(75) Inventors/Applicants (*for US only*): **HUANG, Ru, Chih, C.** [US/US]; 4604 Kemeway, Baltimore, MD 21212 (US). **ABD ELAZEM, Ibrahim, Shawky** [EG/US]; 3601 Greenway, Apt. 103, Baltimore, MD 21218 (US). **CHEN, Hong, Shan** [CN/CN]; 1 Tian-Tan, Beijing 100050 (CN).

(74) Agents: **HUMPHREY, Christopher, M.** et al.; Alston & Bird LLP, Bank of America Plaza, Suite 4000, 101 South Tryon Street, Charlotte, NC 28280-4000 (US).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

(88) Date of publication of the international search report: 24 October 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 02/026726 A3

(54) Title: ANTIVIRAL COMPOSITIONS AND METHODS OF USE

(57) Abstract: Purified antiviral compounds, pharmaceutical formulations containing the compounds, and methods of use of the compounds are provided. The compositions of the invention are isolated antiviral components from plant extracts derived from, for example, *Salvia milriorrhiza*, that find use in the treatment of viral infections, such as by inhibiting the activity of viral integrase. Methods for isolation and purification of the antiviral compounds are additionally provided.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/29906

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07D307/86 A61K31/343 A61P31/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, WPI Data, BEILSTEIN Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 66942 A (GEORGETOWN UNIVERSITY, USA) 29 December 1999 (1999-12-29) abstract; claims 4-17 page 25 -page 45; examples ---	1,8,16, 32
X	H. KOHDA ET AL.: "Isolation of Inhibitors of Adenylate Cyclase from Dan-shen, the Root of Salvia miltiorrhiza" CHEMICAL AND PHARMACEUTICAL BULLETIN, vol. 37, no. 5, 1989, pages 1287-1290, XP002204922 TOKYO JP page 1287, column 2 page 1289, column 2 ---	1,8,32

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 July 2002	26/08/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Paisdor, B

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	CH. J. KELLEY ET AL.: "Polyphenolic Acids of Lithospermum ruderale Dougl. ex Lehm. (Boraginaceae). 1. Isolation and Structure Determination of Lithospermic Acid." JOURNAL OF ORGANIC CHEMISTRY, vol. 40, no. 12, 1975, pages 1804-1815, XP002204923 EASTON US page 1807, column 1 page 1805, column 1 abstract ---	1,8,32
X	H. YAMAMOTO ET AL.: "Caffeic acid oligomers in Lithosermum erythrorhizon cell suspension cultures" PHYTOCHEMISTRY., vol. 53, 2000, pages 651-657, XP002204924 PERGAMON PRESS., GB ISSN: 0031-9422 page 654 -page 656, column 1 ---	1,8,32
X	R. KASIMU ET AL.: "Comparative Study of Seventeen Salvia Plants: Aldose Reductase Inhibitory Activity of Water and MeOH Extracts and Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis of Water Extracts" CHEMICAL AND PHARMACEUTICAL BULLETIN., vol. 46, no. 3, 1998, pages 500-504, XP002204925 PHARMACEUTICAL SOCIETY OF JAPAN. TOKYO., JP ISSN: 0009-2363 page 503 page 500; examples 1,1A ---	1,8,32
X	PATENT ABSTRACTS OF JAPAN vol. 1998, no. 01, 30 January 1998 (1998-01-30) -& JP 09 241157 A (ALPS YAKUHIN KOGYO KK;NANBA TSUNEO), 16 September 1997 (1997-09-16) abstract ---	1,16
X	T. TANAKA ET AL.: "Four New Caffeic Acid Metabolites, Yunnaneic Acids E-H, from Salvia yunnanensis" CHEMICAL AND PHARMACEUTICAL BULLETIN, vol. 45, no. 10, 1997, pages 1596-1600, XP002204926 TOKYO JP page 1596, column 1; example 1 ---	1,8,32

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	T. TANAKA ET AL.: "Magnesium and Ammonium-Potassium Lithospermates B, the Active Principles Having a Uremia-Preventive Effect from Salvia miltiorrhiza" CHEMICAL AND PHARMACEUTICAL BULLETIN., vol. 37, no. 2, 1989, pages 340-344, XP002204927 PHARMACEUTICAL SOCIETY OF JAPAN. TOKYO., JP ISSN: 0009-2363 page 342, column 2 -page 343, column 2 page 340; examples 1-3	1,8,32
X	WO 91 19507 A (CEDARS SINAI MEDICAL CENTER) 26 December 1991 (1991-12-26) abstract; claims 1,11,41	1,8,16
X	US 6 043 276 A (H. K. HAN ET AL.) 28 March 2000 (2000-03-28) abstract; claims page 1 -page 4 page 31 -page 38	1,8,16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 01/29906

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 16-32 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/29906

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9966942 A	29-12-1999	US 6037369 A	14-03-2000
		AU 740698 B2	15-11-2001
		AU 4714999 A	10-01-2000
		CN 1312722 T	12-09-2001
		CZ 20004858 A3	12-09-2001
		EP 1089747 A1	11-04-2001
		NO 20006631 A	26-02-2001
		PL 345086 A1	03-12-2001
		TR 200100450 T2	23-07-2001
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		EP 0491913 A1	01-07-1992
		JP 5500824 T	18-02-1993
		OA 9651 A	15-05-1993
		WO 9119507 A1	26-12-1991
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		EP 1206255 A1	22-05-2002
		WO 0100199 A1	04-01-2001
		AU 740698 B2	15-11-2001
		AU 4714999 A	10-01-2000
		CN 1312722 T	12-09-2001
		CZ 20004858 A3	12-09-2001
		EP 1089747 A1	11-04-2001
		NO 20006631 A	26-02-2001
		PL 345086 A1	03-12-2001
		TR 200100450 T2	23-07-2001
		WO 9966942 A1	29-12-1999

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

CORRECTED VERSION

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 April 2002 (04.04.2002)

PCT

(10) International Publication Number
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(51) International Patent Classification[7]: C07D 307/86, A61K 31/343, A61P 31/18

(21) International Application Number: PCT/US01/29906

(22) International Filing Date: 25 September 2001 (25.09.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/235,425 26 September 2000 (26.09.2000) US

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(54) Title: ANTIVIRAL COMPOSITIONS AND METHODS OF USE

(57) Abstract: Purified antiviral compounds, pharmaceutical formulations containing the compounds, and methods of use of the compounds are provided. The compositions of the invention are isolated antiviral components from plant extracts derived from, for example, *Salvia milriorrhiza*, that find use in the treatment of viral infections, such as by inhibiting the activity of viral integrase. Methods for isolation and purification of the antiviral compounds are additionally provided.

ANTIVIRAL COMPOSITIONS AND METHODS OF USE

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

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FIELD OF THE INVENTION

The present invention relates to compositions and methods for the prevention and treatment of viral infections, particularly retroviral infections.

BACKGROUND OF THE INVENTION

Viruses consist of either double-stranded or single-stranded DNA or RNA enclosed in a protein coat, called a capsid. Some viruses also possess a lipoprotein envelope that, like the capsid, may contain antigenic proteins. Since viruses have no metabolic machinery of their own, they usurp the machinery of their host cell which, depending on the virus, may be a plant, bacterium, or animal cell.

A viral infection begins when a virion comes into contact with a host cell and attaches, or adsorbs, to it. The viral DNA or RNA then crosses the plasma membrane into the cytoplasm and eventually enter into the nucleus. In the case of retrovirus, the viral RNA is reverse transcribed into DNA. Viral DNA is then integrated into the chromosomal DNA of the infected cell. Integration is mediated by an integration protein, integrase. All integrated proviruses are required for the subsequent transcription process which is acted upon by the host cell transcription factors. The integrated DNA is transcribed by the cell's own machinery into mRNA, or replicated and becomes enclosed in a virion. For retrovirus, the integrated DNA is transcribed into RNA that either acts as mRNA or become enclosed in a virion. This completes the virus life cycle.

In the past decade, the emergence of human immunodeficiency virus type 1 (HIV-1) as an important human pathogen has led to a resurgence of scientific interest

in retroviruses. HIV-1 is the primary etiologic agent of AIDS, a fatal disease that results from the gradual destruction of the helper T-cell population in infected individuals. The importance of HIV-1 as a human pathogen has led to its being the major focus of research into lentivirus replication and gene regulation. Indeed, HIV-1 may be viewed as the prototype of not only the lentivirus subgroup but also, more broadly, complex retroviruses in general.

There are an estimated 650,000 to 900,000 people currently living with HIV in the United States, with approximately 40,000 new HIV infections occurring here every year. As of June 1999, 711,344 AIDS cases have been reported in the United States. Since the beginning of the epidemic, 420,201 AIDS deaths have been reported. The scale of the AIDS epidemic demands the development of efficient and affordable AIDS therapeutics.

While HIV-1 relies heavily on the cellular host enzymes for many of the steps required in its replication, the virus carries in its genome the genetic information that leads to the synthesis of its unique retroviral enzymes, such as the three enzymes encoded by its *pol* gene: reverse transcriptase, proteases, and integrase. Effective antiviral agents must inhibit virus-specific replicative events or preferentially inhibit virus-directed rather than host cell-directed nucleic acid or protein synthesis. To date, of the numerous compounds that have already been identified and approved for marketing by the FDA for HIV, only drugs inhibiting the activities of reverse transcriptase and protease inhibitors have been identified. The first drug to be introduced was suramin, a reverse transcriptase inhibitor. Subsequently, AZT and other compounds (zalciabine (ddC), didanosine (ddl), compound Q, ritonavir, etc.) have also been found to possess anti-HIV activity *in vitro*. Specifically, AZT was approved by the FDA in 1987.

Even though the current therapeutic agents are effective in inhibiting the enzymatic activity which is essential for the viral life cycle, the small fraction of remaining viruses unfortunately mutate and continue to replicate even in the presence of these drugs. High rates of replication, viral sequence mutation, and rapid turnover of the viral population are typical traits of retroviruses. These traits are even more

striking in the case of HIV-1. As result, these drugs show little long term benefits in terms of a complete treatment or prevention of HIV-infection. Recent studies have demonstrated that combinatorial therapy against reverse transcriptase (RT) and protease can eliminate a majority of the HIV viruses in T lymphocytes. There is, therefore, need for additional therapeutic agents to be added to the treatment cocktail for viral infections, particularly retroviral infections.

The viral integrase catalyses the integration of the viral DNA into the host DNA, which is an essential step in the viral life cycle. There is no know human homologue to this enzyme and therefore potential inhibitors could be both efficacious and non-toxic. However, drugs targeting integrase have been slow to emerge because of the lack of structural information on this poorly soluble protein. Current search on integrase inhibitor has relied more on empirical testing than on drug design.

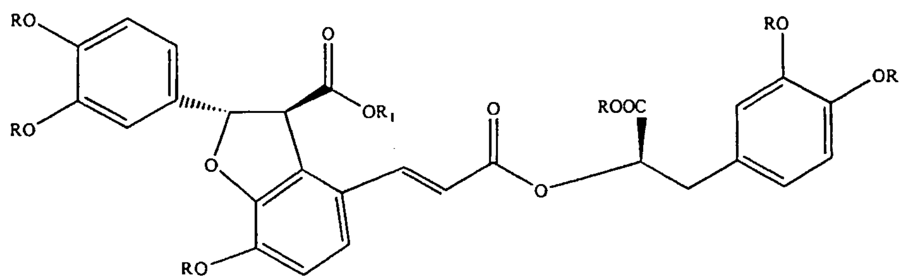
Salvia miltiorrhiza is a traditional Chinese medicinal herb for treatment of cardiovascular and hepatic diseases. Extracts from *S. miltiorrhiza* and its related species exhibit anti-viral and antioxidant activities that are health beneficial. See Meng et al. (1992) *Chung Kuo Chung Hsi I Chieh Ho Tsa Chih* 12, 345-347, 324-35; Xiong (1993) *Chung Kuo Chung Hsi I Chieh Ho Tsa Chih* 13, 33-35, 516-517; U.S. Pat. No. 5,178,865; U.S. Pat. No. 5,411,733; U.S. Pat. No. 6,043,276; International PCT Application 98/24460; Chinese Patent Application Nos. 1,192,922 and 1,192,918. Antiviral agents active against herpes, polio, measles, varicellazoster, cytomegalovirus, DNA viruses and RNA viruses have been described which contain at least one crude drug from the root of *S. miltiorrhiza* Bunge (See European Patent No. 0 568 001 A2). Seven phenolic compounds isolated from the aqueous extract of *S. miltiorrhiza* demonstrate a strong protective action against peroxidative damage to liver microsomes, hepatocytes, or erythrocytes (See Liu, *et al.*, 1992, *Biochem. Pharmacol.* 43, 147-1952). Lithospermic acid B was identified as an active component in an extract of *Salvia miltiorrhiza radix* that was shown to exhibit endothelium-dependent vasodilation in the aorta and may be useful in the treatment of hypertension (See Kamata, *et al.*, 1993, *Gen. Pharmacol.* 24, 977-981). The therapeutic effect of these extract has been attributed in part to the ability of the plant

to accumulate active compounds such as transhinones and phenolic compounds.

Therefore, there remains a need in the art for the identification of additional compounds capable of treating viral infections, particularly compounds that inhibit viral integrase.

SUMMARY OF THE INVENTION

Purified antiviral compounds, pharmaceutical formulations containing the compounds, and methods of use of the compounds are provided. The compositions of the invention are isolated antiviral components from plant extracts that find use in the treatment of viral infections. In particular, it is believed that the compounds of the invention inhibit the activity of viral integrase. The purified compounds are represented by Formula (I):



wherein:

each R is independently H or an alkyl group, such as a C1-C4 alkyl; and

R_1 is H, alkyl, substituted alkyl, aryl or substituted aryl, and pharmaceutically acceptable salts thereof.

Preferably, each R is selected from the group consisting of H and methyl and R_1 is an alkyl group substituted with a substituted phenyl group and a carboxyl group. Preferred substituents on the phenyl ring include one or more hydroxyl groups. Preferably, the purified compounds have a purity of at least about 90%, more preferably at least about 95%, and most preferably at least about 99%.

Methods for the isolation of the antiviral compounds are also provided. The isolation method comprises providing plant material, such as *Salvia miltiorrhiza*, and

extracting an alcohol-soluble fraction therefrom. The roots of the *Salvia* plant are particularly preferred. A group of compounds are precipitated from the alcohol-soluble fraction and separated into an aqueous layer and an organic layer. Thereafter, chromatography can be used to isolate at least one compound of Formula (I) from the organic layer. For example, high performance liquid chromatography (HPLC) can be used in the isolation step.

Methods for treatment or prevention of viral infection, or the disease that it causes it, using the compounds of Formula (I) are also provided. The method involves administering to a population of cells, either *in vitro* or *in vivo*, a therapeutically effective amount of a purified compound of Formula (I) or a pharmaceutically acceptable salt thereof. The compound of Formula (I) may be administered alone or as part of a pharmaceutical composition comprising the purified compound, one or more pharmaceutically acceptable carriers and, optionally, one or more additional antiviral agents. In specific embodiments, methods are provided to treat and/or prevent HIV-1 infection and to treat and/or prevent AIDS by inhibiting the integrase activity of HIV-1. The compositions of the invention find use in inhibiting viral replication in a mammal, particularly a human being.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus described the invention in general terms, reference will now be made to the accompanying drawings, wherein:

Figures 1A and 1B show the inhibition of 3′ processing activities of HIV-1 integrase (IN) in the presence of varying concentrations of P.8s and P.8p fractions;

Figures 2A and 2B show the inhibition of 3′ processing activities of HIV-1 integrase (IN) in the presence of different concentrations of butanol fraction (Bu.M_5);

Figure 3 shows the HPLC chromatogram of the butanol fraction;

Figures 4A and 4B show the HPLC chromatograms of separately pooled $M_5$22 and $M_5$32 peaks from Figure 3 reapplied to the column;

Figures 5A and 5B show the effect of $M_5$22 compound on the inhibition of catalytic activities of HIV-1 integrase (IN) in the presence of different concentrations;

Figures 6A and 6B show the effect of M_532 compound on the inhibition of catalytic activities of HIV-1 integrase (IN) in the presence of different concentrations;

Figures 7A and 7B show the effect of M_522 and M_532 on HIV-1 replication and cell viability; and

Figure 8 shows the inhibition of HIV-1 replication on four different virus strains by using the M_522 compound in H9 cells.

DETAILED DESCRIPTION OF THE INVENTION

The present invention now will be described more fully hereinafter with reference to the accompanying drawings, in which preferred embodiments of the invention are shown. This invention may, however, be embodied in many different forms and should not be construed as limited to the embodiments set forth herein; rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art.

The terms "alkyl," "alkene," and "alkoxy" include straight chain and branched alkyl, alkene, and alkoxy, respectively. The term "lower alkyl" refers to C1-C4 alkyl. The term "alkoxy" refers to oxygen substituted alkyl, for example, of the formulas –OR or –ROR^1, wherein R and R^1 are each independently selected alkyl. The terms "substituted alkyl" and "substituted alkene" refer to alkyl and alkene, respectively, substituted with one or more non-interfering substituents, such as but not limited to, C3-C6 cycloalkyl, e.g., cyclopropyl, cyclobutyl, and the like; acetylene; cyano; alkoxy, e.g., methoxy, ethoxy, and the like; lower alkanoyloxy, e.g., acetoxy; hydroxy; carboxyl; amino; lower alkylamino, e.g., methylamino; ketone; halo, e.g. chloro or bromo; phenyl; substituted phenyl, and the like. The term "halogen" includes fluorine, chlorine, iodine and bromine.

"Aryl" means one or more aromatic rings, each of 5 or 6 carbon atoms. Multiple aryl rings may be fused, as in naphthyl or unfused, as in biphenyl. Aryl rings may also be fused or unfused with one or more cyclic hydrocarbon, heteroaryl, or heterocyclic rings.

"Substituted aryl" is aryl having one or more non-interfering groups as

substituents.

"Non-interfering substituents" are those groups that yield stable compounds. Suitable non-interfering substituents or radicals include, but are not limited to, halo, C_1-C_{10} alkyl, C_2-C_{10} alkenyl, C_2-C_{10} alkynyl, C_1-C_{10} alkoxy, C_7-C_{12} aralkyl, C_7-C_{12} alkaryl, C_3-C_{10} cycloalkyl, C_3-C_{10} cycloalkenyl, phenyl, substituted phenyl, toluoyl, xylenyl, biphenyl, C_2-C_{12} alkoxyalkyl, C_7-C_{12} alkoxyaryl, C_7-C_{12} aryloxyalkyl, C_6-C_{12} oxyaryl, C_1-C_6 alkylsulfinyl, C_1-C_{10} alkylsulfonyl, -$(CH_2)_m$-O-(C_1-C_{10} alkyl) wherein m is from 1 to 8, aryl, substituted aryl, substituted alkoxy, fluoroalkyl, heterocyclic radical, substituted heterocyclic radical, nitroalkyl, -NO_2, -CN, -NRC(O)-(C_1-C_{10} alkyl), -C(O)-(C_1-C_{10} alkyl), C_2-C_{10} thioalkyl, -C(O)O-(C_1-C_{10} alkyl), -OH, -SO_2, =S, -COOH, -NR_2, carbonyl, -C(O)-(C_1-C_{10} alkyl)-CF_3, -C(O)-CF_3, -C(O)NR_2, -(C_1-C_{10} alkyl)-S-(C_6-C_{12} aryl), -C(O)-(C_6-C_{12} aryl), -$(CH_2)_m$-O-$(CH_2)_m$-O-(C_1-C_{10} alkyl) wherein each m is from 1 to 8, -C(O)NR_2, -C(S)NR_2, -SO_2NR_2, -NRC(O)NR_2, -NRC(S)NR_2, salts thereof, and the like. Each R as used herein is H, alkyl or substituted alkyl, aryl or substituted aryl, aralkyl, or alkaryl.

The present invention is drawn to compounds and compositions which find use as antiviral agents. The present invention is also drawn to the method of isolating these compounds from plant extracts and using them to treat a variety of viral infections.

Due to the lack of toxicity and the low IC_{50} values for inhibition of HIV-1 replication and HIV-1 integrase activity exhibited by the compounds of Formula (I) (See Examples), it is believed that purified compounds of Formula (I) are potent antiviral agents and can likely be useful as therapeutic drugs for AIDS, as well as other viruses.

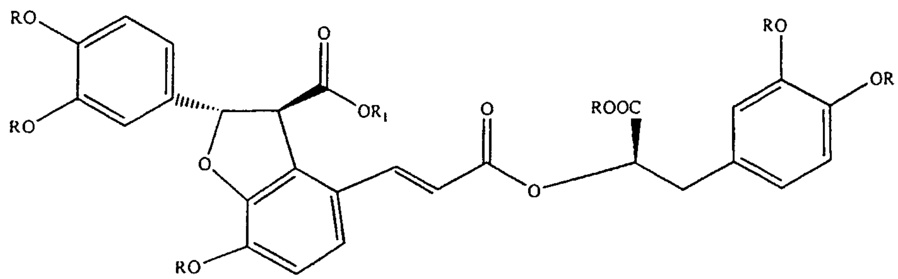
The compounds and compositions of the invention have anti-viral activities. The phrase "antiviral activity" is used herein to mean the effective inhibition of the activity of a virus, including, but not limited to, its propagation or replication. Generally, virus replication includes cell entry, integration into the genome, transcription of the viral genome, translation of viral proteins, post-translational modifications, assembly of virion components, and release. Thus, the compositions of

the invention effectively inhibit at least one aspect of the replication cycle. Assays can be performed to identify the mechanism by which the composition functions to inhibit viral activity. Such assays are well known in the art. See, for example, Lee et al. (1994) *Analytical Biochemistry* 220, 377-383; Lee et al. (1995) *Analytical Biochemistry* 227, 295-301; U.S. Patent application No. 08/365,473; Lee et al. (1995) *Biochemistry* 34, 10205-10214; Lee et al. (1995) *Biochemistry* 34, 10215-10223; Lee and Han (1996) *Biochemistry* 35, 3837-3844.

The present invention encompasses purifying or isolating these antiviral compounds from plants. A purified or isolated compound is substantially free of other compounds. By "substantially free" is intended a purity of at least about 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 99.5%. NMR profiles of samples of M_522 and M_532 compounds have confirmed a purity level of at least about 99 to 99.5%. By "purity" is intended the percentage of the dry weight of the compound of interest divided by the dry weight of the purified fraction containing the compound of interest.

The present invention also encompasses using compositions comprising such isolated compounds for the treatment of a variety of viral infections. By "treatment" is intended the either the reduction of the total number of viral particles, the retardation of viral propagation, or the relief or prevention of symptoms caused by viral infection in a patient following administration of these compositions. Assays can be performed to determine the inhibitory effect of a composition on viral activities, which include, but are not limited to, immunoassays detecting viral antigens, such as viral surface antigens or core antigens. Such assays are well known in the art.

The compositions of the invention comprise a purified compound of Formula (I):

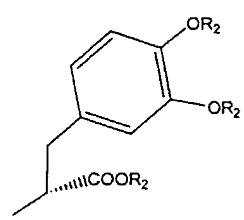


wherein:

each R is independently H or an alkyl group, such as a C1-C4 alkyl; and

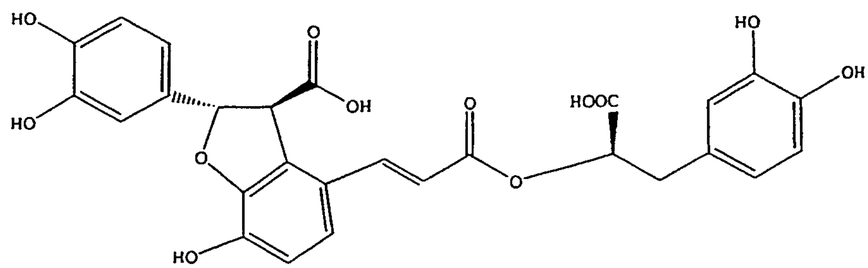
R_1 is H, alkyl, substituted alkyl, aryl or substituted aryl.

Preferred R substituents include H or methyl. Preferred R_1 substituents include alkyl groups substituted with a carboxyl group and a substituted phenyl ring, such as a phenyl ring substituted with one or more hydroxyl groups. In one preferred embodiment, R_1 and each R are H. In another embodiment, each R is H and R_1 is



wherein each R_2 is independently H or alkyl, such as lower alkyl.

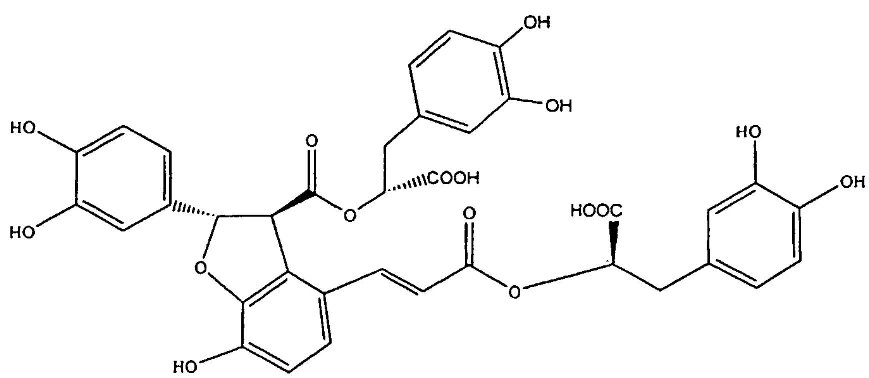
One preferred embodiment, known as lithospermic acid (also referred to as $M_5$22 herein), is shown below. The molecular weight of lithospermic acid is 538.



Lithospermic Acid ($M_5$22)

(4-{2-[l-carboxy-2-(3,4-dihydroxy-phenyl)-ethoxycarbonyl]-vinyl}-2-(3,4-dihydroxy-phenyl)-7-hydroxy-2,3-dihydro-benzofuran-3-carboxylic acid)

Another preferred embodiment, known as lithospermic acid B (also referred to as $M_5$32 herein), is shown below. The molecular weight of lithospermic acid B is 718.



Lithospermic Acid B ($M_5$32)

(4-{2-[l-carboxy-2-(3,4-dihydroxy-phenyl)-ethoxycarbonyl]-vinyl}-2-(3,4-dihydroxy-phenyl)-7-hydroxy-2,3-dihydro-benzofuran-3-carboxylic acid 1-carboxy-2-(3,4-dihydroxy-phenyl)-ethyl ester)

Viral integration is an attractive target because there is no apparent human protein counterpart, the steps involved in proviral integration are similar for all retroviruses, and the structural and functional properties among all types or classes of retroviral integrases are similar. See Khan et al. (1991) *Nucleic Acids Research* 19, 851-860; Thomas and Brady (1997) *Trends in Biotechnology* 15, 167-172. These publications are herein incorporated by reference. Thus, an inhibitor against integrase can be used as an antiviral therapy for a broad range of virus infections including HIV, SIV (simian immunodeficiency virus); MuLV (mouse leukemia virus); and the like.

Assays can be performed to test the inhibitory effect of a composition on the integration of the viral DNA into the host genome. For example, assays to test the ability of the composition to inhibit viral integrase activity are known in the art. Integrase specifically recognizes both ends of the viral DNA and removes two nucleotides from the 3′ ends. The processed viral DNA and integrase then migrate to the nucleus where a viral integrase covalently links the viral genome to host chromosomal DNA, known as strand transfer, forming the provirus. Thus, inhibition of viral integrase results in inhibition of the integration of the viral DNA into the genome of the infected cells and the replication of the viral DNA. Thus, the level of integrase activity can be assayed by measuring the degree to which the ends of viral DNA or fragments containing viral DNA sequences are processed. It is recognized that the sequence of the DNA fragment used in the assay will vary according to the recognition specificity of the particular viral integrase that is being assayed. See Chow (1997) *Methods* 12, 306-317; Kulkosky et al. (1995) *Virology* 206, 448-456; Katzman and Katz (1999) *Advances in Virus Research* 52, 371-395. These publications are herein incorporated by reference. A compound that inhibits viral integrase activity can reduce the level of integrase activity by at least about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99%.

The compositions of the invention can be used alone or in combination with other antiviral agents that inhibit the same or different aspects of the viral replication cycle (i.e., integration, viral entry, proviral transcription, viral replication, or viral assembly). Such agents include, but are not limited to, zidovudine (AZT), didanosine

(ddI), stavudine (d4T), zalcitabine (ddC), amantadine interferon, ribavirin, rimantadine, and NDGA derivatives (See Gnabre, *et al.*, 1995, *Proc. Natl. Acad. Sci.*, USA 92, 11239-11243; Hwu, et al., 1998, *J. Med. Chem.*, 41, 2994-3000; Chen, et al., 1998, *J. Med. Chem.*, 41, 3001-3007; U.S. Patent No. 6, 214,874 B).

The methods and compositions of the invention are useful against a wide array of viruses. For example, both simple and complex retroviruses are encompassed by the present invention. Members of those taxonomic divisions are set forth in Table 1.

Table 1: Major taxonomic divisions among retroviruses

Category	Subgroup	Prototype	Other examples
Simple retroviruses	C-type retroviruses Group A	RSV	ALV, ASV
	C-type retroviruses Group B	MLV	FeLV,MSV,SNV, REV,SSV
	B-type retroviruses	MMTV	
	D-type retroviruses	MPMV	SRV-1
Complex retroviruses	Lentiviruses	HIV-1	HIV-2,SIV,visna virus,FIV,EIAV
	T-cell leukemia viruses	HTLV-1	TLV-II,STLV, BLV
	Spumaviruses	HSRV	SFV,BFV

Abbreviations: RSV, Rous sarcoma virus; ALV, avian leukemia virus; ASV, avian sarcoma virus; FeLV, feline leukemia virus; MSV, murine sarcoma virus; SNV, spleen necrosis virus; REV, reticuloendotheliosis virus; SSV, simian sarcoma virus; MMTV, mouse mammary tumor virus; MPMV, Mason-Pfizer monkey virus; SRV-1, simian retrovirus type 1; STLV, simian T-cell leukemia virus; BFV, bovine foamy virus

The methods and compositions of the present invention are also useful in the treatment of diseases and/or clinical symptoms resulting from a viral infection. Such viral infections include, but are not limited to, infections caused by the complex group of retroviruses including all lentiviruses, spumaviruses as well as HTLV-1 and related viruses, which are responsible for diseases such as acquired immunodeficiency syndrome (AIDS) and T-cell leukemias (the human T lymphotrophic virus I, HTLV-I).

The antiviral compositions and compounds of the invention can be purified or partially purified from plants and plant extracts. Of particular interest are plants from the genus *Salvia*, such as *S. miltiorrhiza*, *S. officinalis*, *S. splendens*, *S. lyrata*, *urticifolia*, *S. farinace*, particularly *Salvia miltiorrhiza*. Of particular interest are extracts obtained from plant roots.

Methods are readily available for the partial purification or complete purification of the antiviral compound of the invention. Such methods include, for example, centrifugation, dialysis, solvent extraction (using solvent systems including methanol, dichloromethane, propanol, ethanol, butanol, etc.), precipitation, column separations, chromatography (liquid, anion exchange, cation exchange, thin layer, affinity, hydrophobic interaction, gel filtration, reverse phase, high performance liquid, etc.), mass spectrometry, and the like. Such methods can be used in any sequence or combinations. Generally, the antiviral compounds of the invention can be separated based on chemical and physical properties, such as solvent solubility, molecular size, charge, polarity, and hydrophobicity. For example, after an initial purification using dialysis, extraction and chromatography, tandem mass spectrometry (MS/MS) can be performed on the sample extract. MS/MS is utilized when mixtures contain components of the same molecular weight. Likewise, LS/MS/MS is a powerful tool for characterizing samples with large numbers of components.

Methods for such procedures are readily available in the prior art. See, for example, *Perry's Chemical Engineers' Handbook*, Sixth Ed. (Robert H. Perry and Don Green (eds.)) McGraw-Hill, Inc. (1984); *Practical HPLC methodology and applications* (Brian A. Bidlingmeyer) 1992 Wiley, New York; *A Practical guide to HPLC detection* (edited by Donald Parriott) 1993 Academic Press, Inc., San Diego, CA; *Solvent extraction in analytical chemistry* (George H. Morrison and Henry Freiser) 1957 Wiley New York; *Solvent extraction in biotechnology: recovery of primary and secondary metabolites* (Karl Schugerl) 1994 Springer-Verlag Berlin, New York; *Interpretation of mass spectra of organic compounds* (Mynard C. Hamming and Norman G. Foster) 1972 Academic Press, Inc. New York; Youngquist et al. (1995) *J. Am. Chem. Soc.* 117: 3900-3906; Dunayevskiy et al (1995) *Anal.*

Chem. 67: 2906-2915; Brummel et al. (1996) *Anal. Chem.* 68: 237-242; Metzger et al. (1994) *Analytical Biochemistry* 219: 261-277; Brummel et al. (1994) *Science* 264: 399-402; *Methods in Enzymology*, Vol. 182, *Guide to Protein Purification*, edited by Murray P. Deutscher 1990, Academic Press, Inc. San Diego, CA; herein incorporated by reference.

In a preferred method, the compounds of the invention are isolated or purified from plant material, such as *Salvia miltiorrhiza*. The preferred method comprises extracting an alcohol-soluble fraction from the plant material, such a root material. A group of compounds are then precipitated from the alcohol-soluble fraction and separated into an aqueous layer and an organic layer, such as a butanol-soluble layer. Thereafter, chromatography techniques can be used to isolate at least one compound of Formula (I) from the organic layer. For example, high performance liquid chromatography (HPLC) can be used in the isolation step.

At each stage of the purification process, the resulting fractions can be tested for antiviral activity. In this manner, partially purified extracts, extracts containing at least the active compound of Formula (I), a partially purified compound of Formula (I), or an isolated compound of Formula (I) having antiviral activity can be obtained. Antiviral activity can be tested using standard techniques such as the integrase assay set forth in the Experimental Section or others known in the art.

After purification of antiviral compounds by the methods noted above, various methods are available in the art for the determination of the structure of the isolated compounds; for example, mass spectroscopy and nuclear magnetic resonance (NMR), and the like.

The purified antiviral compounds can be administered in various compositions to a population of cells that are infected with the virus. The population of cells can be cultured *in vitro*, or found *in vivo* in a living organism, more particularly a mammal. *In vitro*, the population of cells can be adherent cells or cells in suspension. *In vivo*, the population of cells can be from any organ or combination of organs of the body of the organism.

The antiviral compositions can be used for the treatment of patients infected with a virus or viruses, when the compositions of the present invention has an inhibitory effect on the activity of such virus. All viral infections in patients are potentially treatable by the composition in the present invention.

The antiviral compositions can be formulated according to known methods to prepare pharmaceutically useful compositions, such as by admixture with a pharmaceutically acceptable carrier vehicle. Suitable vehicles and their formulation are described, for example, in *Remington's Pharmaceutical Sciences* (16th ed., Osol, A. (ed.), Mack, Easton PA (1980)). In order to form a pharmaceutically acceptable composition suitable for effective administration, such compositions will contain an effective amount of the antiviral compound, either alone, or with a suitable amount of carrier vehicle.

Additional pharmaceutical methods may be employed to control the duration of action. Controlled release preparations may be achieved by the use of polymers to complex or absorb the antiviral compositions. The controlled delivery may be exercised by selecting appropriate macro molecules (for example, polyesters, polyamino acids, polyvinyl pyrrolidone, ethylene-vinylacetate, methylcellulose, carboxymethylcellulose, or protamine sulfate). The rate of drug release may also be controlled by altering the concentration of such macromolecules.

Another possible method for controlling the duration of action comprises incorporating the therapeutic agents into particles of a polymeric substance such as polyesters, polyamino acids, hydrogels, poly(lactic acid) or ethylene vinylacetate copolymers. Alternatively, it is possible to entrap the therapeutic agents in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerization, for example, by the use of hydroxymethyl cellulose or gelatin-microcapsules or poly(methylmethacrylate) microcapsules, respectively, or in a colloid drug delivery system, for example, liposomes, albumin, microspheres, microemulsions, nanoparticles, nanocapsules, or in macroemulsions. Such teachings are disclosed in *Remington's Pharmaceutical Sciences* (1980).

It is contemplated that the inhibitory compositions of the present invention will be administered to an individual in therapeutically effective amounts. That is, in an amount sufficient to inhibit the replication and/or propagation of the target virus. The effective amount of the inhibitory composition will vary according to the weight, sex, age, and medical history of the individual. Other factors which influence the effective amount may include, but are not limited to, the severity of the patient's condition, the severity of viral infection, the stability of the antiviral compound, the kinetics of interaction between the virus and the antiviral compound, previous exposure to the inhibitory compound, kidney or other disease, etc. An effective amount can reduce the level of virus activity by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%, preferably about 95%, 96%, 97%, or 98%, more preferably about 99%. Typically, a therapeutically effective amount will range from about 0.1 mg to about 300 mg per kg of body weight per day.

The pharmaceutically prepared inhibitory compositions of the invention may be provided to a patient by means well known in the art. Such means of introduction include oral means, intranasal means, subcutaneous means, intramuscular means, intravenous means, intraarterial means, or parenteral means.

The antiviral compounds of the present invention may be dissolved in any physiologically tolerated liquid in order to prepare an injectable bolus. It is generally preferable to prepare such a bolus by dissolving the molecule in normal saline.

Thus, the present invention provides a method of treating AIDS in a subject, comprising administering to the subject an effective amount of a compound of the present invention, such as a compound of Formula (I). The administering step can comprise administering an effective amount of the compound in a pharmaceutically acceptable carrier.

For the purposes of AIDS therapy, a compound of Formula (I) is administered to the subject in an amount sufficient to inhibit HIV-1 from further infecting other cells. However, the therapeutically effective dosage of any specific compound will vary somewhat from compound to compound, patient to patient, and will depend upon the condition of the patient and the route of delivery. When administered conjointly

with other pharmaceutically active agents, even less of the compounds of Formula (I) may be therapeutically effective. The compound of Formula (I) may be administered once or several times a day. The duration of the treatment may be once per day for a period of from two to three weeks and may continue for a period of months or even years. The daily dose can be administered either by a single dose in the form of an individual dosage unit or several smaller dosage units or by multiple administration of subdivided dosages at certain intervals.

The compounds of Formulas (I) may be administered per se or in the form of a pharmaceutically acceptable salt. When used in medicine, the salts of the compounds of Formulas (I) should be both pharmacologically and pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare the free active compound or pharmaceutically acceptable salts thereof and are not excluded from the scope of this invention. Such pharmacologically and pharmaceutically acceptable salts can be prepared by reaction of a compound of Formula (I) with an organic or inorganic acid, using standard methods detailed in the literature. Examples of useful salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicyclic, p-toluenesulfonic, tartaric, citric, methanesulphonic, formic, malonic, succinic, naphthalene-2-sulphonic and benzenesulphonic, and the like. Also, pharmaceutically acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium, or calcium salts of the carboxylic acid group.

Thus the present invention also provides pharmaceutical formulations or compositions, both for veterinary and for human medical use, which comprise the a compound of Formula (I) or a pharmaceutically acceptable salt thereof with one or more pharmaceutically acceptable carriers thereof and optionally any other therapeutic ingredients, such as other chemotherapeutic agents for AIDS. The carrier(s) must be pharmaceutically acceptable in the sense of being compatible with the other ingredients of the formulation and not unduly deleterious to the recipient thereof.

The compositions includes those suitable for oral, rectal, topical, nasal, ophthalmic, or parenteral (including intraperitoneal, intravenous, subcutaneous, or

intramuscular injection) administration. The compositions may conveniently be presented in unit dosage form and may be prepared by any of the methods well known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier that constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier or both, and then, if necessary, shaping the product into desired formulations.

Compositions of the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets, tablets, lozenges, and the like, each containing a predetermined amount of the active agent as a powder or granules; or a suspension in an aqueous liquor or non-aqueous liquid such as a syrup, an elixir, an emulsion, a draught, and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine, with the active compound being in a free-flowing form such as a powder or granules which is optionally mixed with a binder, disintegrant, lubricant, inert diluent, surface active agent or dispersing agent. Molded tablets comprised with a suitable carrier may be made by molding in a suitable machine.

A syrup may be made by adding the active compound to a concentrated aqueous solution of a sugar, for example sucrose, to which may also be added any accessory ingredient(s). Such accessory ingredients may include flavorings, suitable preservatives, an agent to retard crystallization of the sugar, and an agent to increase the solubility of any other ingredient, such as polyhydric alcohol, for example, glycerol or sorbitol.

Formulations suitable for parental administration conveniently comprise a sterile aqueous preparation of the active compound, which can be isotonic with the blood of the recipient.

Nasal spray formulations comprise purified aqueous solutions of the active agent with preservative agents and isotonic agents. Such formulations are preferably adjusted to a pH and isotonic state compatible with the nasal mucous membranes.

Formulations for rectal administration may be presented as a suppository with a suitable carrier such as cocoa butter, or hydrogenated fats or hydrogenated fatty carboxylic acids.

Ophthalmic formulations are prepared by a similar method to the nasal spray, except that the pH and isotonic factors are preferably adjusted to match that of the eye.

Topical formulations comprise the active compound dissolved or suspended in one or more media such as mineral oil, petroleum, polyhydroxy alcohols or other bases used for topical formulations. The addition of other accessory ingredients as noted above may be desirable.

Further, the present invention provides liposomal formulations of the compounds of Formula (I) and salts thereof. The technology for forming liposomal suspensions is well known in the art. When the compound of Formula (I) or salt thereof is an aqueous-soluble salt, using conventional liposome technology, the same may be incorporated into lipid vesicles. In such an instance, due to the water solubility of the compound or salt, the compound or salt will be substantially entrained within the hydrophilic center or core of the liposomes. The lipid layer employed may be of any conventional composition and may either contain cholesterol or may be cholesterol-free. When the compound or salt of interest is water-insoluble, again employing conventional liposome formation technology, the salt may be substantially entrained within the hydrophobic lipid bilayer that forms the structure of the liposome. In either instance, the liposomes that are produced may be reduced in size, as through the use of standard sonication and homogenization techniques. The liposomal formulations containing the compounds of Formula (I) or salts thereof, may be lyophilized to produce a lyophilizate which may be reconstituted with a pharmaceutically acceptable carrier, such as water, to regenerate a liposomal suspension.

Pharmaceutical formulations are also provided which are suitable for administration as an aerosol, by inhalation. These formulations comprise a solution or suspension of the desired compound of Formula (I) or a salt thereof or a plurality of solid particles of the compound or salt. The desired formulation may be placed in a

small chamber and nebulized. Nebulization may be accomplished by compressed air or by ultrasonic energy to form a plurality of liquid droplets or solid particles comprising the compounds or salts.

In addition to the aforementioned ingredients, the compositions of the invention may further include one or more accessory ingredient(s) selected from the group consisting of diluents, buffers, flavoring agents, binders, disintegrants, surface active agents, thickeners, lubricants, preservatives (including antioxidants) and the like.

Having now generally described this invention, the same will be better understood by reference to certain specific examples which are included herein for purposes of illustration only, and are not intended to be limiting of the invention, unless specified.

EXPERIMENTAL

The plant, *Salvia miltiorrhiza*, (SM), has been recorded in the Chinese traditional medical book "Ben-cao-gan-mu" with the function of "to activate blood and to resolve stagnant." It showed multiple pharmacological activities both *in vitro* and *in vivo*. Its preparations have been used safely to treat cardiovascular and liver diseases for generations. The herb is widely distributed in China with different species in different districts.

In the appended examples, purification and testing of active anti-HIV compounds from crude extracts of *Salvia miltiorrhiza* roots is described. Two potent, non-toxic HIV-1 integrase inhibitors, $M_5 22$ and $M_5 32$, were isolated using high performance liquid chromatography (HPLC). Both are pure compounds that showed strong anti-HIV activity in infected H9 cells.

The following cells and virus strains were obtained from the AIDS Research and Reference Regent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases: H9 cells, virus strains of HIV-1_{IIIB}A17, a variant resistant to RT nonnucleotide inhibitors and HIV-$1_{L10R/M46I/L63P/V82T/I84V}$, a protease inhibitor resistant virus, as well as viral strains HTLV-III_{mn}, HIV-1_{RTMF} and HTLV-III_B. Virus-infected

H9 cells were cultured in the presence of different concentrations of M_522 and M_532 and viral replication was assayed using the HIV/p24 monoclonal antibody assay.

The HIV-1 integrase assay utilized in testing described below used a DNA substrate consisting of a DNA sequence derived from the U5 end of HIV-1 LTR. It was prepared by annealing oligonucleotide U5V1 (5'-GACCCTTTTAGTCAGTGT GGAAAATC TCTAGCAGT) with its complementary strand U5V2 (3'-CTGGGAAAATCAGTCACACCTTTTAGAGATCGTCA). The U5V1 strand was labeled at the 5'-end with [γ-^{32}P] ATP using T4 polynucleotide kinase as described previously (Kamata et al., 1994). The standard reaction mixture included reaction buffer (40 mM HEPES, pH 7.5, 20 mM $MnCL_2$, 60 mM NaCl, 20 mM DTT and 0.1% Nonidet - P40) and HIV-1 integrase (NIH AIDS Research and Reference Reagent Program). To assay for 3'-end processing, the labeled substrate was incubated with the reaction mixture for 60 minutes at 37°C. An equal volume of stop solution (95% formamide, 30 mM EDTA, 0.1 % xylene cyanol, 0.1 % bromophenol blue) was added to each reaction and the samples were heated to 95°C for 5 minutes to denature the DNA. The samples were then fractionated by electrophorsis on a 15% denaturing polyacrylamide gel. The 3'-end processing activity was monitored by the appearance of a radioactive oligonucleotides product (33nt), shortened by two nucleotides from the original substrate (35nt). The products were visualized by autoradiography and quantitation was carried out by phosphorimaging.

Example 1

Isolation and Testing of Propanol Soluble (P.8s) and Precipitate (P.8p) Fractions from *Salvia miltiorrhiza roots*

Root powder (1.5 kg) of *Salvia miltiorrhiza* (supplied by Jiang Su Danhua Group Co., China) was extracted three times with 4.5 liters of 70% methanol for 24 hours each time with vigorous shaking. The three extracts (70 S fraction) were combined, evaporated to dryness and dissolved in 2.0 liters of 25% NH_40H. The extract solution was then precipitated with four volumes of 1-propanol and the precipitate was washed three times with 5.0 liters of P.8 buffer (Propanol: H_20:

NH_4OH, 80:15:5). Both the propanol soluble (P.8s) and precipitate (P.8p) fractions were tested for anti-HIV integrase activity.

The results of testing are shown in Figures 1A and 1B. The 3'-end processing product catalyzed by HIV-1 integrase (33nt) and the DNA substrate (35nt) are shown. In Figure 1A, different concentrations of supernatant fraction (P.8s) were tested with HIV-1 integrase: lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3-5) integrase with 0.25, 0.5 and 1.0 μg/ml of P.8s fraction; lane 6, 33 nucleotide marker. In Figure 1B, the 3'-end processing activity of HIV-1 integrase was assayed with the precipitate fraction (P.8p): lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3-12) integrase with 0.006, 0.012, 0.025, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 μg/ml of P.8p and lane 13, 33 nucleotide marker. The P.8 precipitate (P.8p), which represented approximately 34% of the root material, showed potent anti-HIV integrase activity in the 3' cleavage activity assay with an IC_{50} of 0.1 μg/ml while the P.8 soluble fraction (P.8s), representing 66% of the starting material was relatively less active (IC_{50} = 1 μg/ml).

To evaluate the potential clinical value of the P.8p fraction, intraperitoneal acute and subacute toxicity tests in mice were conducted for seven days, where P.8p was given once each day for the acute test and twice each day for the subacute test. The toxicity tests for the P.8p fraction were conducted in kunming mice (body weight, 20±1 gm) over a period of seven days. For the acute toxicity test, groups of 10 mice were given doses of 0.45, 0.69, 0.98, 1.60 and 2.00 g/kg once each day and for the subacute toxicity test, mice were given doses of 0.1, 0.2, 0.4 g/kg twice each day.

The number of mouse deaths were recorded daily and LD_{50} was calculated on the 7th day (see Table 2 below). The results showed that the LD_{50} of the P.8p fraction was 1.2 g/ kg for the acute toxicity test and 0.18 g/ kg for the subacute toxicity test. The P.8p extract showed cumulative toxicity in mice when given intraperitoneally in multiple doses. Thus, although the P.8p fraction contained highly active HIV integrase inhibitors, it also contained materials toxic to animals that should be removed.

Table 2 – Acute and Subacute Toxicity Tests of the P.8p Fraction in Mice

Toxicity Test	Administration Route	Dosage (g/kg)	No. mice	Mortality (7th day)	LD_{50} (g/kg) (7th day)
Acute *	IP	2.00	10	10/10	1.20
		1.60	10	8/10	
		0.98	10	4/10	
		0.69	10	0/10	
		0.45	10	0/10	
Subacute**	IP	0.40	10	10/10	0.18
		0.20	10	6/10	
		0.10	10	0/10	

* P.8p was given once each day

** P.8p was given twice each day

Example 2

Isolation and Testing of Butanol Fraction ($Bu.M_5$) from P.8p Fraction

To separate these toxic materials from the antiviral components, a second purification step was employed. For further purification of the P.8p fraction, 50 grams were dissolved in 200 ml of NAA buffer (7 % acetonitrile, O.1M of ammonium acetate; 0.5 % acetic acid) and the cleared NAA fraction was next mixed with 200 ml of 1-butanol and shaken vigorously. The mixture was partitioned into an NAA layer and a butanol layer, which were collected separately. The butanol ($Bu.M_5$) fraction was dried, redissolved in 5% methanol, cleared by centrifugation and then dried again.

The anti-HIV integrase activity of $Bu.M_5$ was tested. The 3'-end processing product catalyzed by HIV-1 integrase (33nt) and the DNA substrate (35nt) are shown in Figures 2A and 2B. In Figure 2A, different concentrations of the $Bu.M_5$ fraction were tested with HIV-1 integrase: lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3-9) integrase with 0.08, 0.16, 0.3, 0.6, 1.25, 2.5 and 5.0 μg/ml of $Bu.M_5$. In Figure 2B, the percent inhibition of HIV-1 integrase activity with

increasing concentrations of Bu.M_5 fraction is illustrated. The IC_{50} of this fraction is 0.6 μg/ml.

Thus, anti-HIV integrase activity was evident (yield < 1 %) in the combined butanol extracts (Bu.M_5).

The toxicity of the Bu.M_5 fraction was tested in C57bl/6 mice by tail vein injections of the Bu.M_5 fraction (2mg/ml in 0.9% NaCl) over a period of seven days at a daily dose of 10 mg/kg, 20 mg/kg and 30 mg/kg, using two mice per dosage group. The Bu.M_5 fraction showed no toxicity in C57bl/6 mice, with no weight loss observed after tail vein injections of 10mg-30mg/kg each day for seven days. Thus, it is believed that the butanol-soluble fraction of the P.8p fraction would also be useful as an antiviral agent.

Example 3

Purification, Identification and Testing of HIV-integrase Active Compounds from Bu.M_5 Fraction

A. Isolation of Active Compounds

High performance liquid chromatography (HPLC) was used to further purify the Bu.M_5 fraction and to isolate pure anti-HIV compounds from *S. miltiorrhiza* roots. A limited amount (100 mg) of butanol fraction (Bu.M_5) was used for each run. HPLC was performed on Waters liquid chromatograph equipped with two 510 pumps and a 996 photodiode array detector. The extract solution was separated and analyzed by using a 250 x 10 mm. preparative C18 (8μm) column with the mobile phase consisting of 5% methanol. The flow rate was 1.0 ml/min and the elution was monitored at a wavelength of 254 nm to facilitate the detection of the different compounds. Figure 3 is a HPLC chromatogram of the Bu.M_5 fraction. Many peaks were resolved at different retention times. The activity of each one was tested against HIV-1 integrase activity to identify the active compounds.

Two major peaks with retention times of 22.4 minutes and 31.4 minutes ($M_5$22 and $M_5$32) were well separated from a large, exceedingly complex mixture of unresolved compounds. These two major peaks were separately pooled and then

reapplied to the column. Figures 4A and 4B are the HPLC chromatograms of separately pooled M_522 and M_532 peaks from Fig. 3 that were reapplied to the column. Figure 4A shows a single and major peak (M_522) was eluted at 22.4 retention time. Figure 4B shows one major peak (M_532) that eluted at about 31.4 retention time. Both M_522 and M_532 were found to be 99%+ pure compounds by NMR analysis.

Approximately 500 runs were made to isolate M_522 and M_532. The yield for M_522 and M_532 from the Bu.M_5 fraction was approximately 13% and 26%, respectively. An overall yield from the initial *Salvia miltiorrhiza* roots of 0.018 % and 0.038 % for M_522 and M_532 was obtained. A brief summary of the purification and the HPLC profiles for compounds M_522 and M_532 are shown in Table 3 below.

Table 3 - A summary of the Purification of HIV-1 Inhibitors from *S. miltiorrhiza* Roots

	Fraction	Amount (g)	Yield (%)
	S. miltiorrhiza roots	1500	
Step I	P.8p	516	34.4
Step II	Bu.M_5	2.2	0.15
Step III	HPLC pure compounds:		
	M_522	0.277	0.018
	M_532	0.572	0.038

B. Identification of Isolated Compounds

The structures of M_522 and M_532 were identified by NMR and MS analysis using known structural standards for comparison (Tanaka et al., 1989). M_522 was identified as lithospermic acid: (4-{2-[l-carboxy-2-(3,4-dihydroxy-phenyl)-ethoxycarbonyl]-vinyl}-2-(3,4-dihydroxy-phenyl)-7-hydroxy-2,3-dihydro-benzofuran-3-carboxylic acid). M_532 was identified as lithospermic acid B: (4-{2-[l-carboxy-2-(3,4-dihydroxy-phenyl)-ethoxycarbonyl]-vinyl}-2-(3,4-dihydroxy-phenyl)-7-hydroxy-2,3-dihydro-benzofuran-3-carboxylic acid 1-carboxy-2-(3,4-dihydroxy-phenyl)-ethyl ester).

C. Efficacy Testing of Isolated Compounds

The anti-HIV efficacy of the two isolated compounds was analyzed by their effect on the 3' processing activity assay and their inhibition of HIV in cultured H9 cells. The inhibitory data indicate that the two compounds are highly potent against HIV integrase, with IC_{50} of 0.45 μg/ml, 0.83 μM for $M_5$22 and 0.35 μg/ml, 0.45 μM for $M_5$32. These results are illustrated in Figures 5 and 6.

Figs. 5A and 5B illustrate the effect of $M_5$22 on the inhibition of HIV-1 integrase (IN) activity in the presence of different concentrations. In Figure 5A, the 3'-end processing product catalyzed by HIV-1 integrase (33nt) and the DNA substrate (35nt) are shown: lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3-9) integrase with 0.15, 0.3, 0.6,1.25, 2.5, 5.0, and 10 μg/ml of $M_5$22 and lane 10, 33 nucleotide marker. Figure 5B is a quantitation of assay results by phosphorimager showing a dose-response curve for the inhibition of HIV-1 integrase activity by the $M_5$22 compound. The IC_{50} of $M_5$22 is 0.45 μg/ml, 0.83 μM.

Figs. 6A and 6B illustrate the effect of $M_5$32 on the inhibition of HIV-1 integrase (IN) activity in the presence of different concentrations. In Figure 6A, the 3'- end processing product catalyzed by HIV-1 integrase (33nt) and the DNA substrate (35nt) are shown: lane 1, without integrase (IN); lane 2, integrase (IN) alone; lanes (3-9) integrase with 0.15 0.3, 0.6,1.25, 2.5, 5.0, and 10 μg/ml of $M_5$32 and lane 10, 33 nucleotide marker. Figure 6B is a quantitation of assay results by phosphorimager showing a dose-response curve for the inhibition of HIV-1 integrase activity by the $M_5$32 compound. The IC_{50} of $M_5$32 is 0.35 μg/ml, 0.45 μM.

The inhibitory effect of $M_5$22 and $M_5$32 against HIV replication was further examined using HIV-1_{IIIB}A17, a variant resistant to RT nonnucleotide inhibitors and HIV-$1_{L10R/M46I/L63P/V82T/I84V}$, a protease inhibitor resistant virus, as testing viruses in the presence of a variety of drug concentrations in infected H9 cells. By using the HIV/p24 monoclonal antibody assay, the IC_{50} for $M_5$22 and $M_5$32 against HIV replication were found to be 1.5μg/ml, 2.7 μM for $M_5$22 and 1 μg/ml, 1.39 μM for $M_5$32.

Figure 7A graphically illustrates the effect of $M_5$22 and $M_5$32 on HIV-1 replication. Figure 7A illustrates the dose-dependent inhibition of HIV-1 replication in H9 cells infected with HIV-1_{IIIB}A17 and HIV-$1_{L10R/M461/L63P/V82T/184V}$ viruses in the presence of different drug concentrations of $M_5$22 and $M_5$32. Viral replication was measured using the HIV/p24 monoclonal antibody assay and the IC_{50} values for inhibition by $M_5$22 and $M_5$32 against HIV replication were calculated as 2.7 μM and 1.39 μM respectively.

D. Cytotoxicity of Isolated Compounds

The cytotoxicity of $M_5$22 and $M_5$32 drugs against H9 cells was analyzed using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Sigma Chemical Co.) (Uckun et al., 1998). Briefly, exponentially growing H9 cells were seeded onto 96-well plates at a density of $3x10^4$ cells/well and incubated for 24 h at 37°C prior to drug exposure. On the day of treatment, a series Of $M_5$22 and $M_5$32 concentrations (1.25, 2.5, 5, 10, 20, 40, 80, and 160 μg/ ml) were used to test their cytotoxicity in H9 cells. Quadruplicate wells were used for each treatment. The cells were incubated with $M_5$22 and $M_5$32 for 4 days at 37°C in a humidified 5% CO_2 atmosphere. To each well, 50 μl of MTT (1mg/ml final concentration) was added and the plates were incubated at 37°C for 4 h to allow MTT to form formazan crystals by reacting with metabolically active cells. The formazan crystals were solubilized with DMSO. The absorbance of each well was measured in a microtiter reader at optical density 540 nm. The reaction is specific, no significant amounts of formazan can be detected with dead cells. We found that the cells remained viable even at the highest concentrations (CC_{100}) of $M_5$22 and $M_5$32 tested (160 μg/ml, 297 μM for $M_5$22 and 160 μg/ml, 223 μM for $M_5$32), indicating that both compounds are non-toxic. These results are presented graphically in Figure 7B.

Example 4

M$_5$22 and M$_5$32 Inhibit the Replication of Four Different Virus Strains in Human H9 Cells

Inhibitory activity of M$_5$22 and M$_5$32 against the replication of four different HIV-1 strains was tested in this example. Two of the strains were drug-resistant virus isolates (HIV-1$_{RTMF}$ resistant against AZT and HIV-1$_{IIIB}$A17 variant resistant against inhibition by RT nonnucleotide inhibitors) and the other two were primary isolates (HTLV-III$_{MN}$ and HTLV-III$_B$).

On the day before infection, H9 cells were subcultured at 1-2 x 10^5 cells/ml and on the day of the infection, cells were pelleted by centrifugation at room temperature for 10 minutes. The pellet was then resuspended with 1 ml of each strain according to the virus titer. The mixtures were incubated at 37 °C in CO_2 incubator for 2 hours. The cells were then washed two times with PBS and then two times with the culture medium (RPMI 1640). The cells were resuspended with fresh medium and plated in 96 well plates. Every 3-4 days after infection, cells were sub-cultured and supernatant was saved to assay for virus production.

The activity of M$_5$22 against the four strains were tested at various drug concentrations of 80, 40, 20, 10, 5, 2.5, 1.25, and 0 μg/ml. After 8 days, the infection of the cells was detected by using HIV-1 p24 antigen assay, which is an enzyme immunoassay (EIA, or enzyme-linked immunosorbent assay) developed for detection and quantitation of the HIV-1 p24 core protein. The percentage of HIV-1 inhibition achieved by the different concentrations of M$_5$22 are shown in Table 4 below. The IC$_{50}$ of M$_5$22 for HIV-1$_{RTMF}$, HIV-1 III$_B$ A17, HTLV-III$_B$ and HTLV-III$_{MN}$ were 2.7 μg/ml, 1.5 μg/ml, 11 μg/ml, and 9 μg/ml, respectively. These results are illustrated graphically in Figure 8.

Table 4

Inhibition of Four Strains HIV-1 Replication by $M_5$22 Drug

% Inhibition

	HIV-1 $_{RTMF}$	HTLV-III $_{MN}$	HIV-III$_B$A17 VARIANT	HTLV-III$_B$
00. μg/ml	0.0	0.0	0.0	0.0
1.25 μg/ml	26.2	7.7	51.2	31.4
2.5 μg/ml	47.7	21.8	53.6	42.1
5.0 μg/ml	78.6	30.0	76.8	45.9
10.0 μg/ml	83.4	51.9	85.0	47.3
20.0 μg/ml	95.5	72.1	90.2	62.0
40.0 μg/ml	98.2	77.3	100.0	82.4
*80.0 μg/ml	100.0	88.4	100.0	94.5

* The concentration of 80.0 μg/ml is equal to 148.5 μM

The same type of test was conducted to examine the effect of $M_5$32 against the same four strains of HIV-1. The percentage of HIV-1 inhibition achieved by the different concentrations of $M_5$32 are shown in Table 5 below. The IC_{50} of $M_5$32 for HIV-1$_{RTMF}$, HTLV-III$_{MN}$, HIV-1 III$_B$ A17 and HTLV-III$_B$ were 5.6 μg/ml, 5.5 μg/ml, 5.0 μg/ml, and 11 μg/ml, respectively.

Table 5

Inhibition of Four Strains HIV-1 Replication by $M_5$32 Drug

% Inhibition

	HIV-1 $_{RTMF}$	HTLV-III $_{MN}$	HIV-III$_B$A17 VARIANT	HTLV-III$_B$
00. μg/ml	0.0	0.0	0.0	0.0
1.25 μg/ml	20.8	8.3	2.0	4.7
2.5 μg/ml	38.7	45.7	21.8	16.1
5.0 μg/ml	44.2	47.0	50.0	20.1
10.0 μg/ml	73.6	57.2	53.6	34.5
20.0 μg/ml	90.4	87.0	97.5	90.8
40.0 μg/ml	95.0	90.0	97.7	96.2
*80.0 μg/ml	98.3	94.1	98.4	96.8

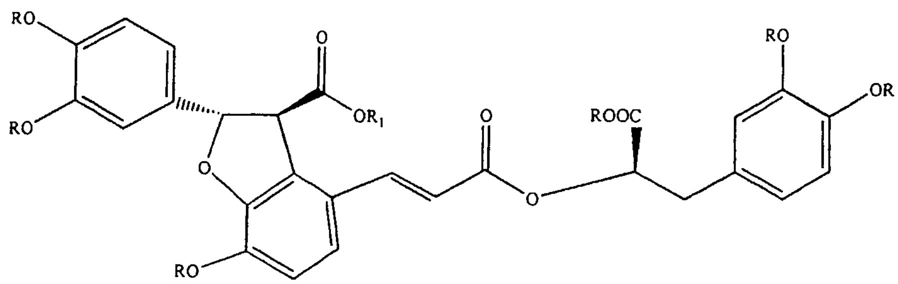
* The concentration of 80.0 μg/ml is equal to 111.5 μM

All publications and patent applications mentioned in this specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Many modifications and other embodiments of the invention will come to mind to one skilled in the art to which this invention pertains having the benefit of the teachings presented in the foregoing descriptions and the associated drawings. Therefore, it is to be understood that the invention is not to be limited to the specific embodiments disclosed and that modifications and other embodiments are intended to be included within the scope of the appended claims. Although specific terms are employed herein, they are used in a generic and descriptive sense only and not for purposes of limitation.

THAT WHICH IS CLAIMED:

1. A purified compound having the Formula (I):



wherein each R is independently H or an alkyl group and R_1 is H, alkyl, substituted alkyl, aryl or substituted aryl, or a pharmaceutically acceptable salt thereof.

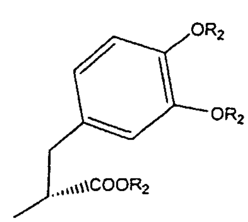
2. The compound of Claim 1, wherein each R is H.

3. The compound of Claim 1, wherein said purified compound has a purity of at least about 90%.

4. The compound of Claim 1, wherein said purified compound has a purity of at least about 99%.

5. The compound of Claim 1, wherein R_1 is substituted alkyl.

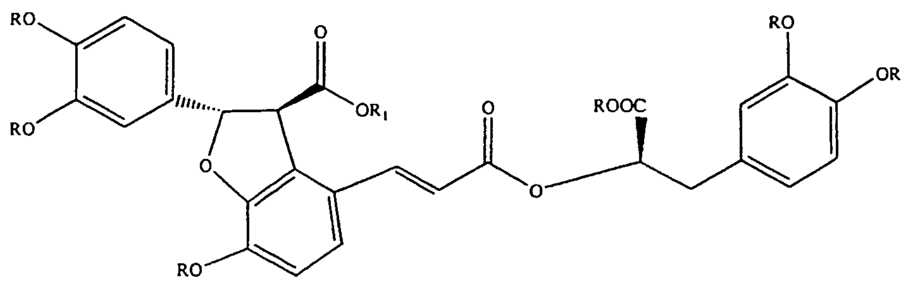
6. The compound of Claim 1, wherein R_1 is:



wherein each R_2 is independently H or alkyl.

7. The compound of Claim 6, wherein each R_2 is H.

8. A pharmaceutical composition, comprising a purified compound having the Formula (I):



wherein each R is independently H or an alkyl group and R_1 is H, alkyl, substituted alkyl,

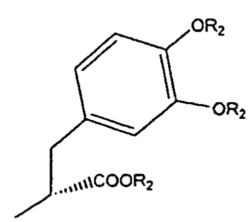
aryl or substituted aryl, or a pharmaceutically acceptable salt thereof; and

a pharmaceutically acceptable carrier.

9. The pharmaceutical composition of Claim 8, wherein each R is H.

10. The pharmaceutical composition of Claim 8, wherein R_1 is substituted alkyl.

11. The pharmaceutical composition of Claim 8, wherein R_1 is:



wherein each R_2 is independently H or alkyl.

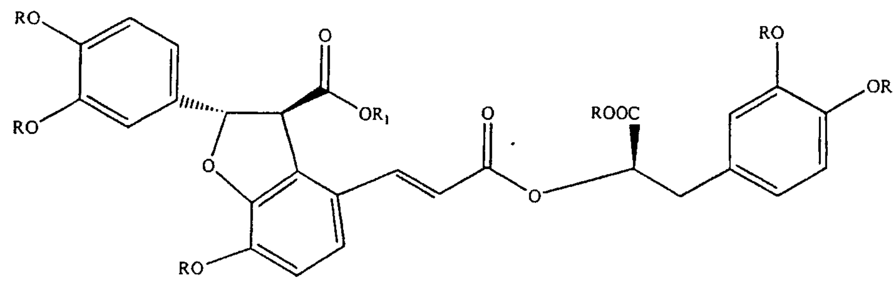
12. The pharmaceutical composition of Claim 11, wherein each R_2 is H.

13. The pharmaceutical composition of Claim 8, wherein said purified compound has a purity of at least about 90%.

14. The pharmaceutical composition of Claim 8, wherein said purified compound has a purity of at least about 99%.

15. The pharmaceutical composition of Claim 8, further comprising at least one additional antiviral agent.

16. A method of treating a virus in a population of cells infected with a virus, comprising administering to the population of cells a therapeutically effective amount of a purified compound of Formula (I):

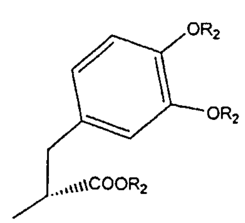


wherein each R is independently H or an alkyl group and R_1 is H, alkyl, substituted alkyl, aryl or substituted aryl, or a pharmaceutically acceptable salt thereof.

17. The method of Claim 16, wherein each R and R_1 are H.

18. The method of Claim 16, wherein R_1 is substituted alkyl.

19. The method of Claim 16, wherein R_1 is:



wherein each R_2 is independently H or alkyl.

20. The method of Claim 19, wherein each R_2 is H.

21. The method of Claim 16, wherein the virus is a retrovirus.

22. The method of Claim 16, wherein the virus is HIV-1.

23. The method of Claim 22, wherein the strain of the HIV-1 virus is selected from the group consisting of HIV-1_{RTMF}, HIV-1 III_B A17 variant, HTLV-III_{MN}, HTLV-III_B, and HIV-$1_{L10R/M46I/L63P/V82T/I84V}$.

24. The method of claim 16, wherein the purified compound reduces the activity of a viral integrase.

25. The method of Claim 16, wherein the purified compound is administered at a concentration of at least about 0.1 μg/ml.

26. The method of Claim 16, wherein the purified compound is purified from a plant extract.

27. The method of Claim 26, wherein the plant extract is from the genus *Salvia*.

28. The method of Claim 27, wherein the plant extract is *Salvia miltiorrhiza*.

29. The method of Claim 16, wherein the purified compound is administered in a pharmaceutically acceptable carrier.

30. The method of Claim 16, wherein the population of cells are cultured *in vitro*.

31. The method of Claim 16, wherein the population of cells are *in vivo*.

32. The method of Claim 16, wherein the purified compound is administered in combination with at least one additional antiviral agent.

33. A method of purifying a compound of Formula (I) from a plant, said method comprising:

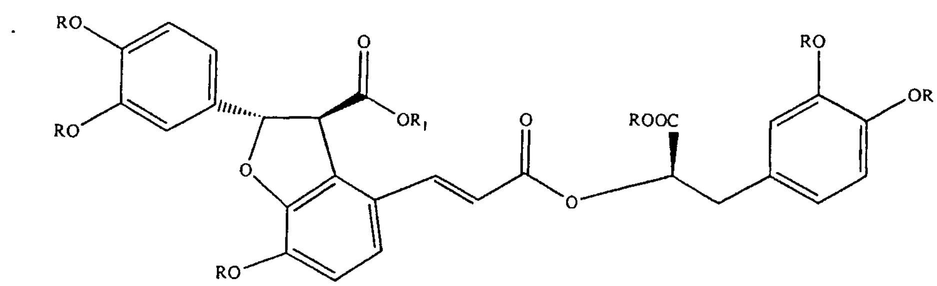
providing plant material from the genus *Salvia*;

extracting an alcohol-soluble fraction from the plant material;

precipitating a group of compounds from the alcohol-soluble fraction;

separating the precipitated compounds into an aqueous layer and an organic layer; and

isolating at least one compound from the organic layer using chromatography, the compound being of Formula (I):

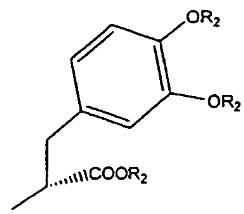


wherein each R is independently H or an alkyl group and R_1 is H, alkyl, substituted alkyl, aryl or substituted aryl.

34. The method of Claim 33, wherein each R and R_1 are H.

35. The method of Claim 33, wherein R_1 is substituted alkyl.

36. The method of Claim 33, wherein R_1 is:



wherein each R_2 is independently H or alkyl.

37. The method of Claim 36, wherein each R_2 is H.

38. The method of Claim 33, wherein the plant material is *Salvia miltiorrhiza*.

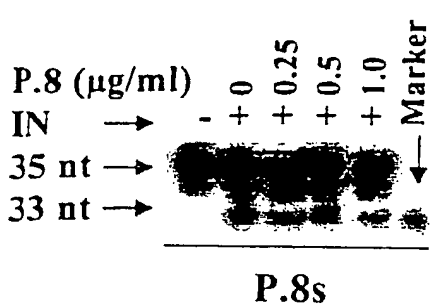


FIG. 1A.

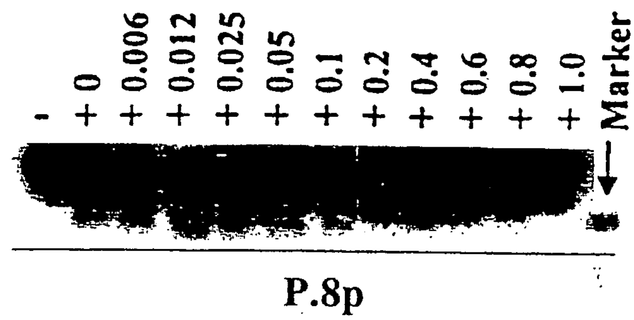


FIG. 1B.

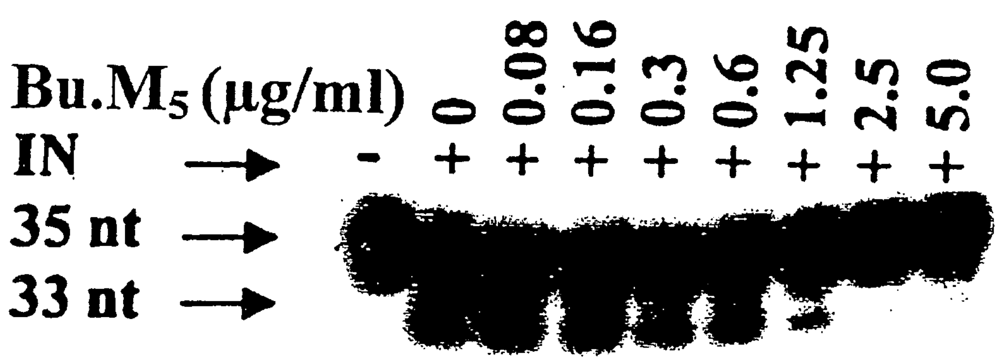


FIG. 2A.

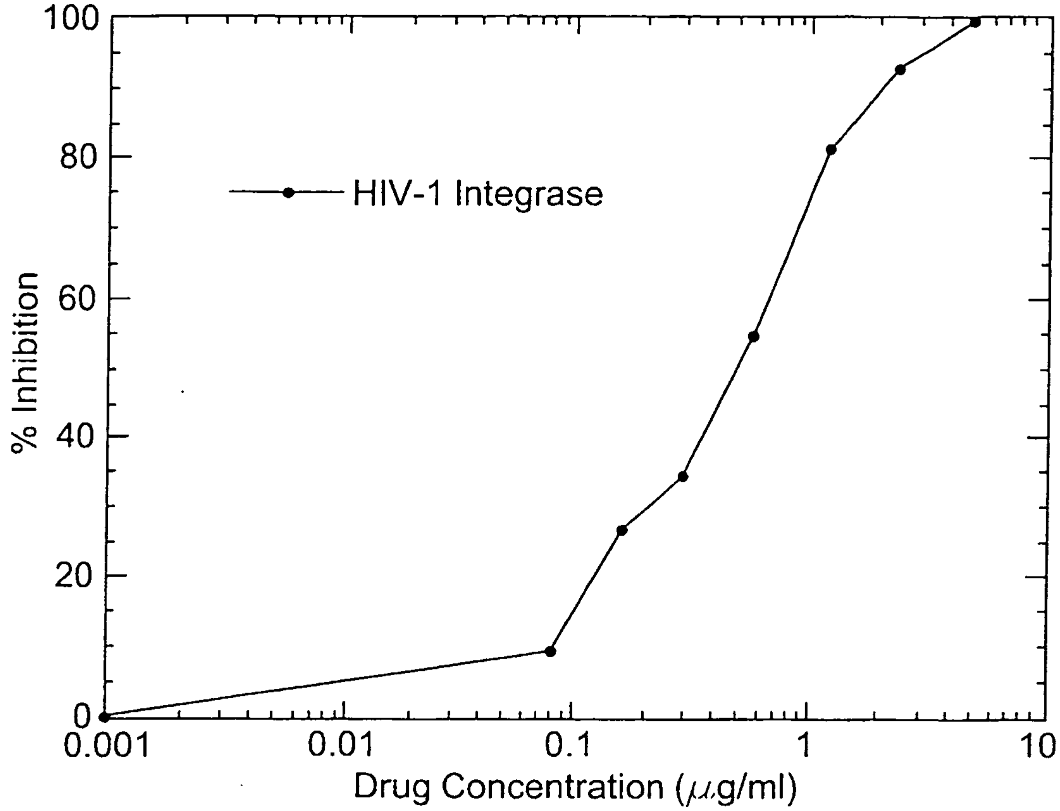

100
80
60
40
20
0
% Inhibition
HIV-1 Integrase
0.001
0.01
0.1
1
10
Drug Concentration (μg/ml)

FIG. 2B.

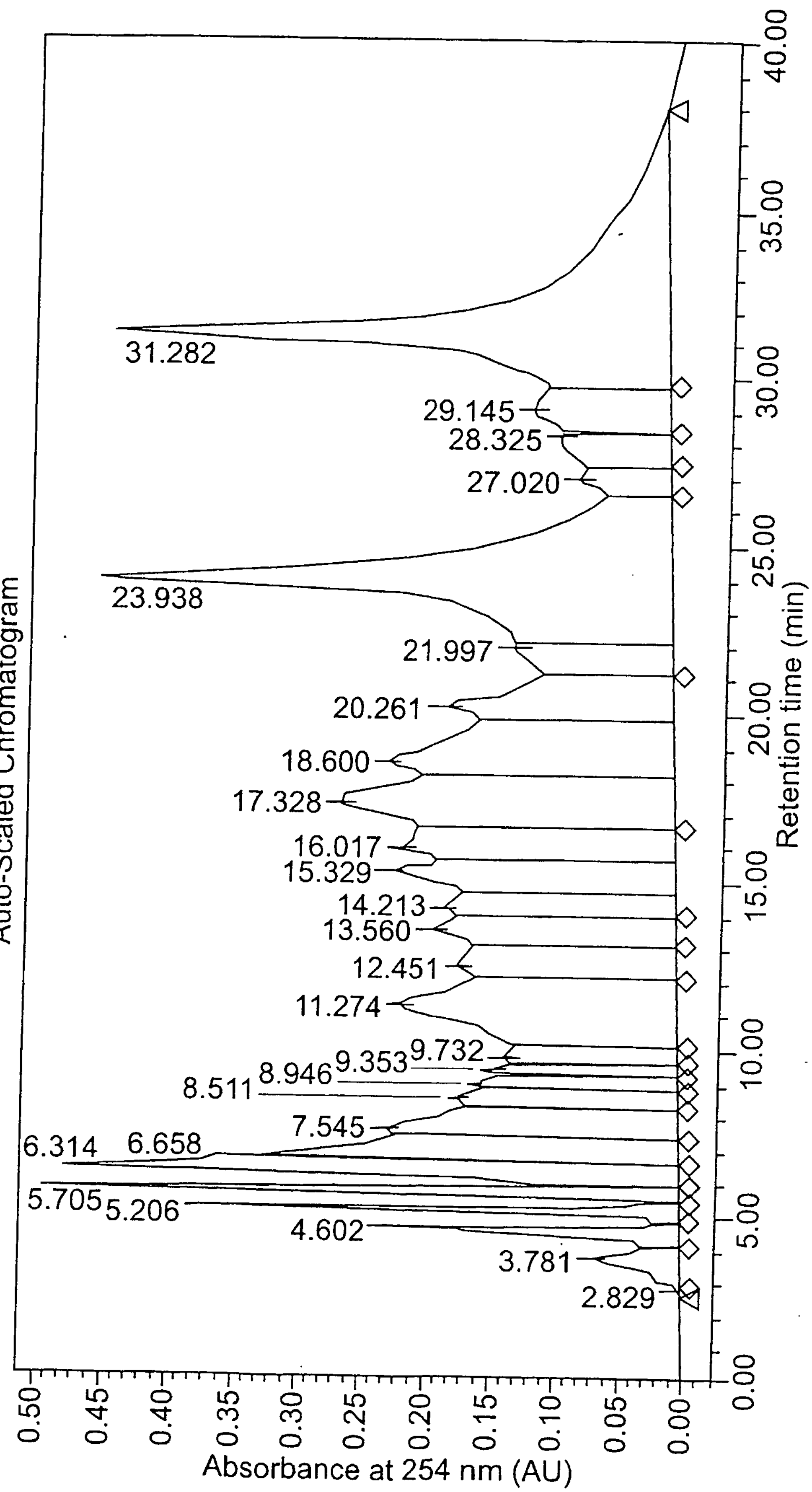

Auto-Scaled Chromatogram
Absorbance at 254 nm (AU)
Retention time (min)
0.50
0.45
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
31.282
29.145
28.325
27.020
23.938
21.997
20.261
18.600
17.328
16.017
15.329
14.213
13.560
12.451
11.274
9.732
9.353
8.946
8.511
7.545
6.314
6.658
5.705
5.206
4.602
3.781
2.829

FIG. 3.

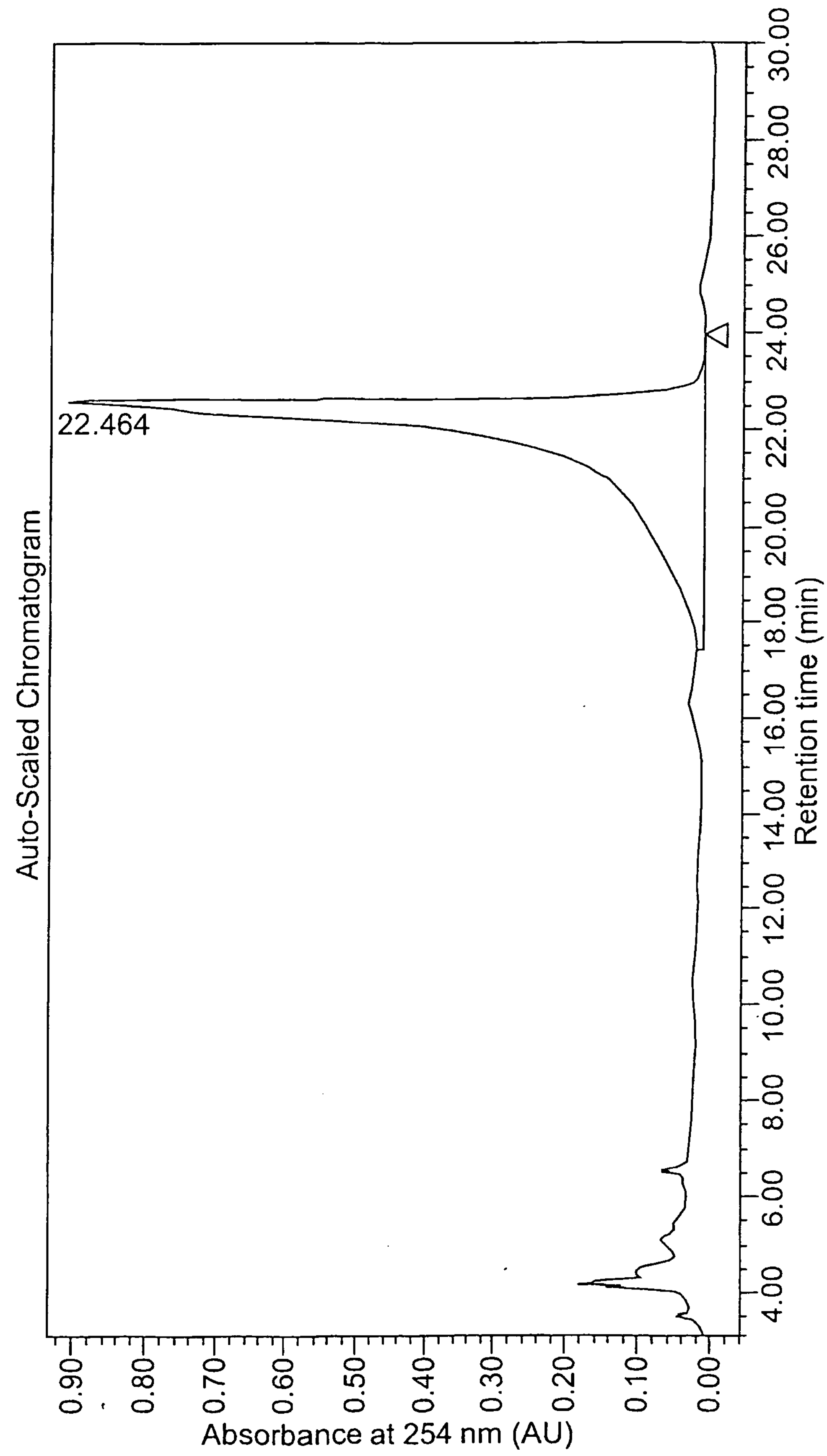

Auto-Scaled Chromatogram
22.464
Absorbance at 254 nm (AU)
0.90
0.80
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
Retention time (min)
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
20.00
22.00
24.00
26.00
28.00
30.00

FIG. 4A.

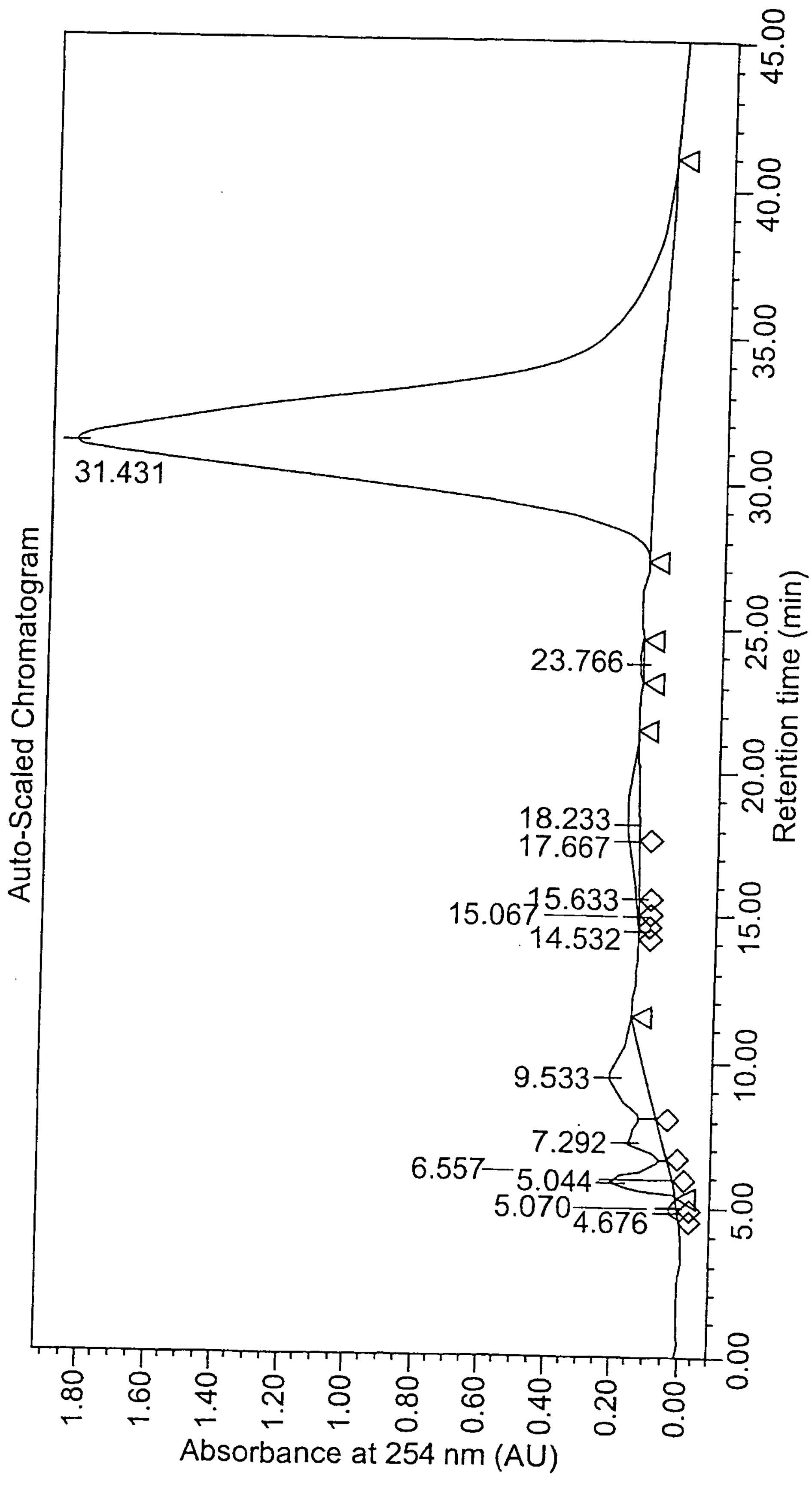

Auto-Scaled Chromatogram
31.431
23.766
18.233
17.667
15.633
15.067
14.532
9.533
7.292
6.557
5.044
5.070
4.676
Absorbance at 254 nm (AU)
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Retention time (min)
0.00
5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00

FIG. 4B.

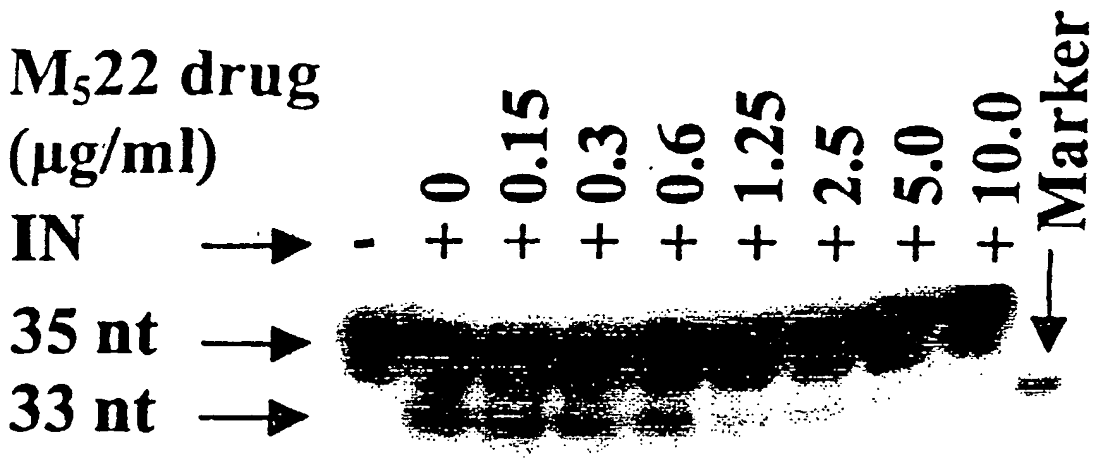


FIG. 5A.

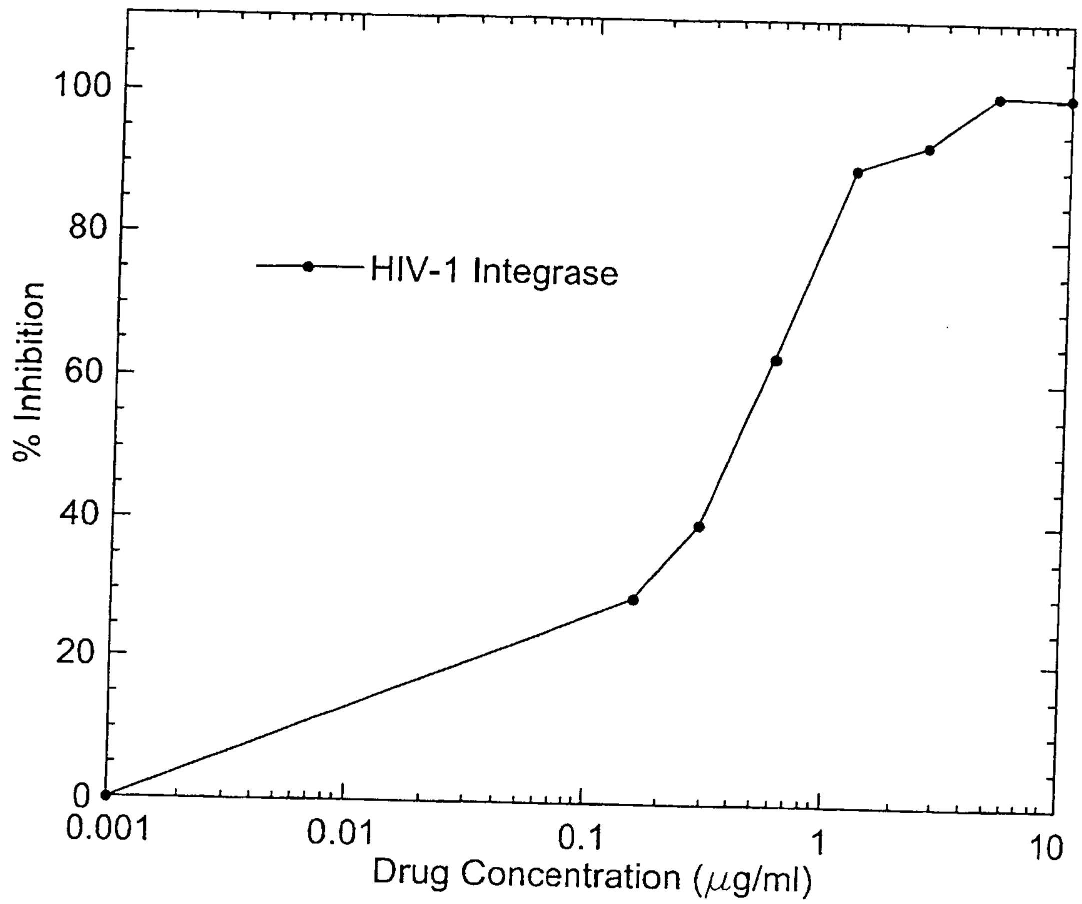

100
80
60
40
20
0
% Inhibition
HIV-1 Integrase
0.001
0.01
0.1
1
10
Drug Concentration (μg/ml)

FIG. 5B.

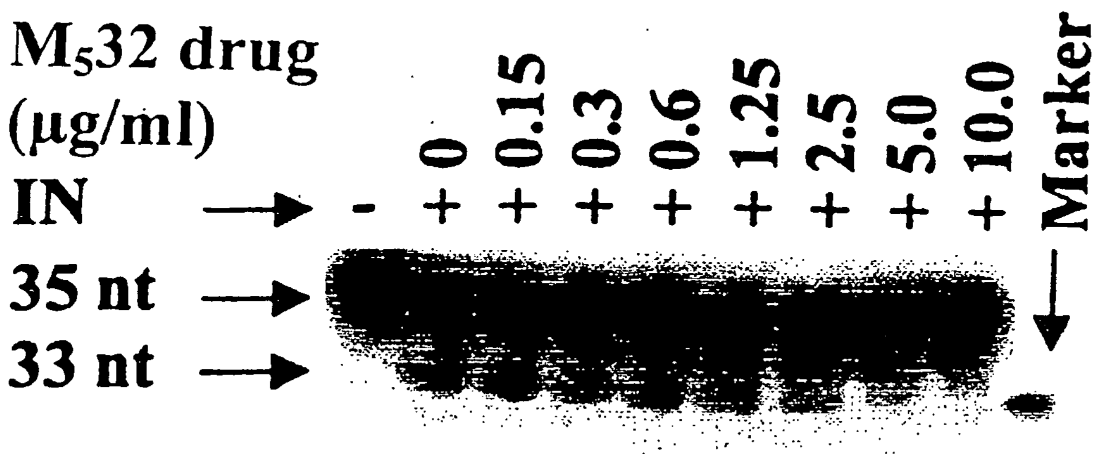


FIG. 6A.

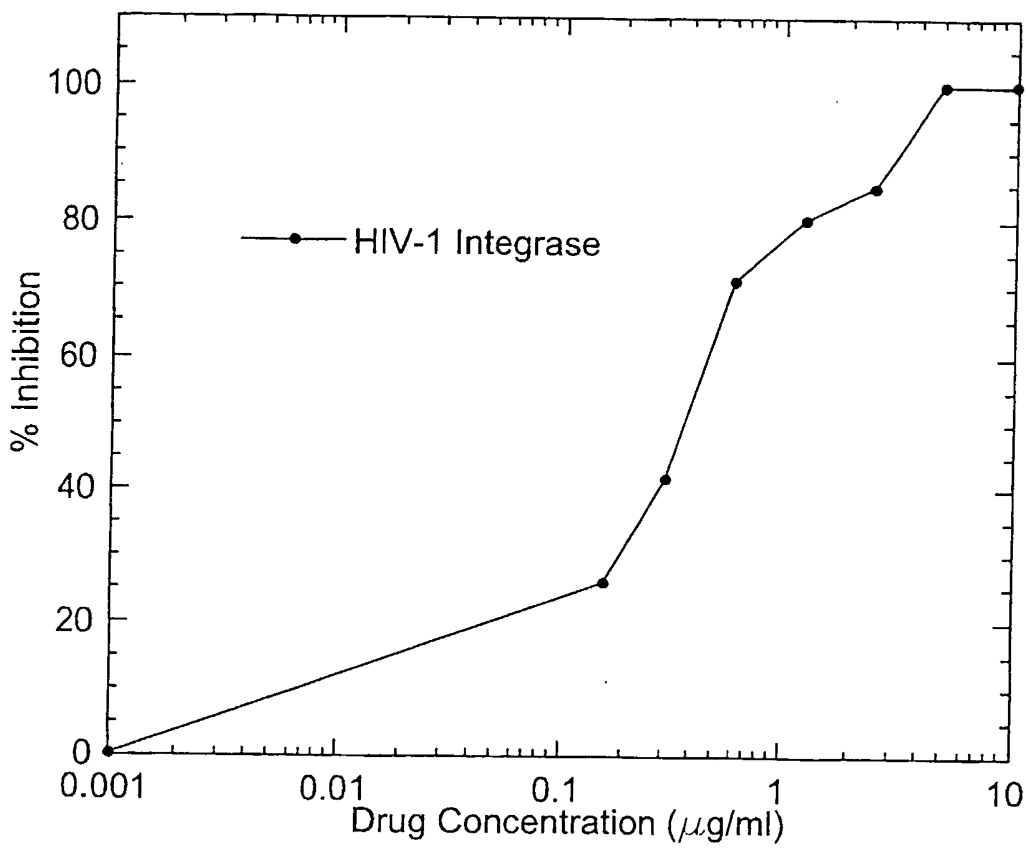

100
80
60
40
20
0
% Inhibition
HIV-1 Integrase
0.001
0.01
0.1
1
10
Drug Concentration (μg/ml)

FIG. 6B.

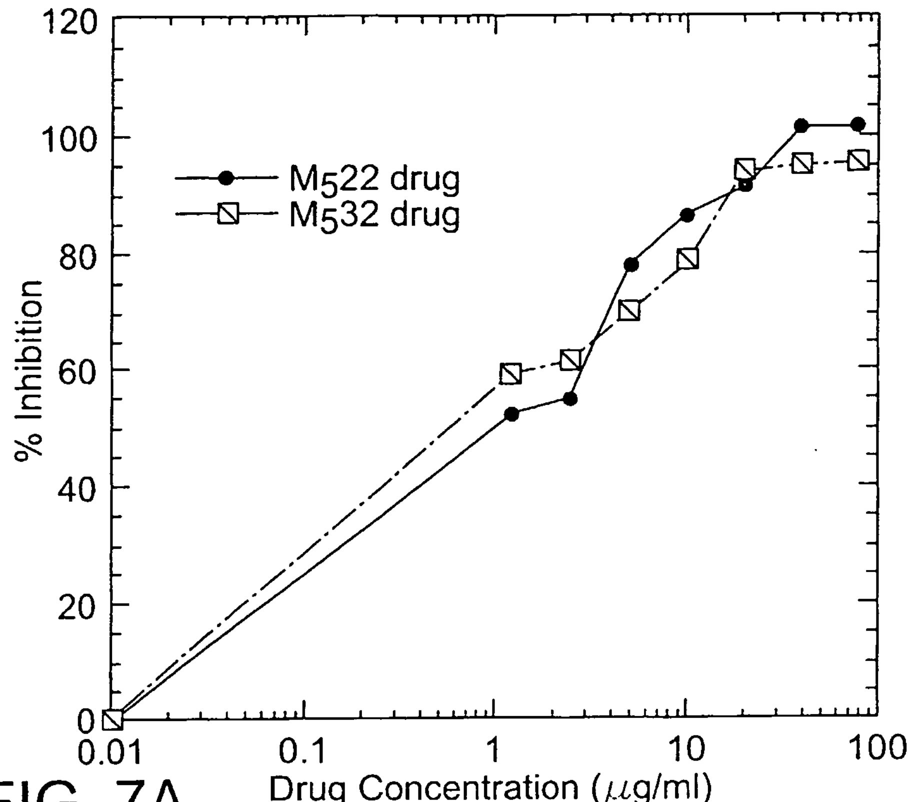

120
100
80
60
40
20
0
% Inhibition
M522 drug
M532 drug
0.01
0.1
1
10
100
Drug Concentration (μg/ml)

FIG. 7A.

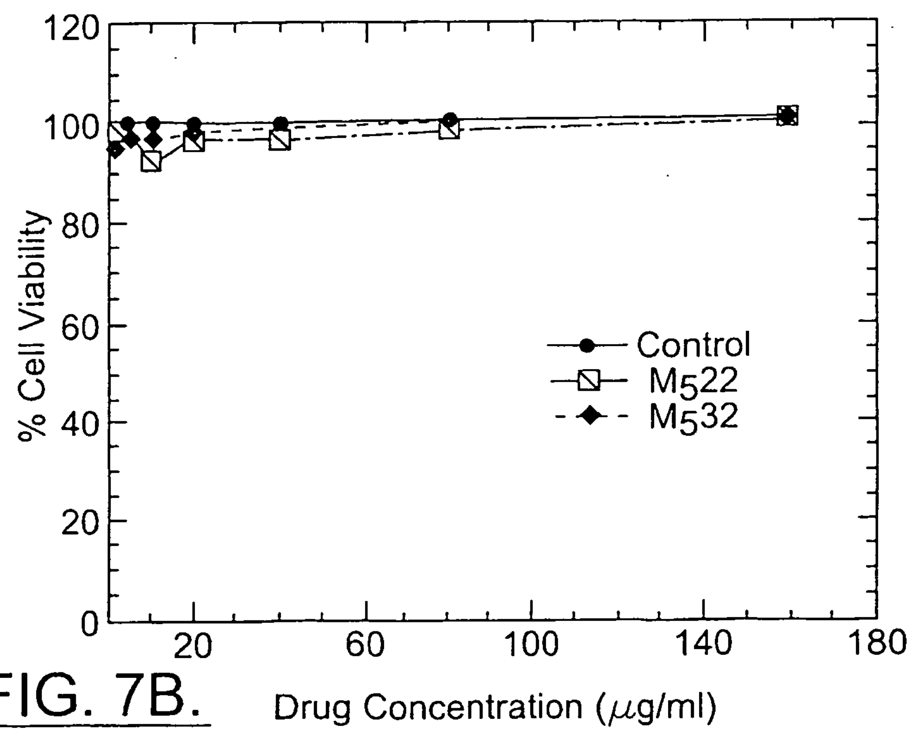
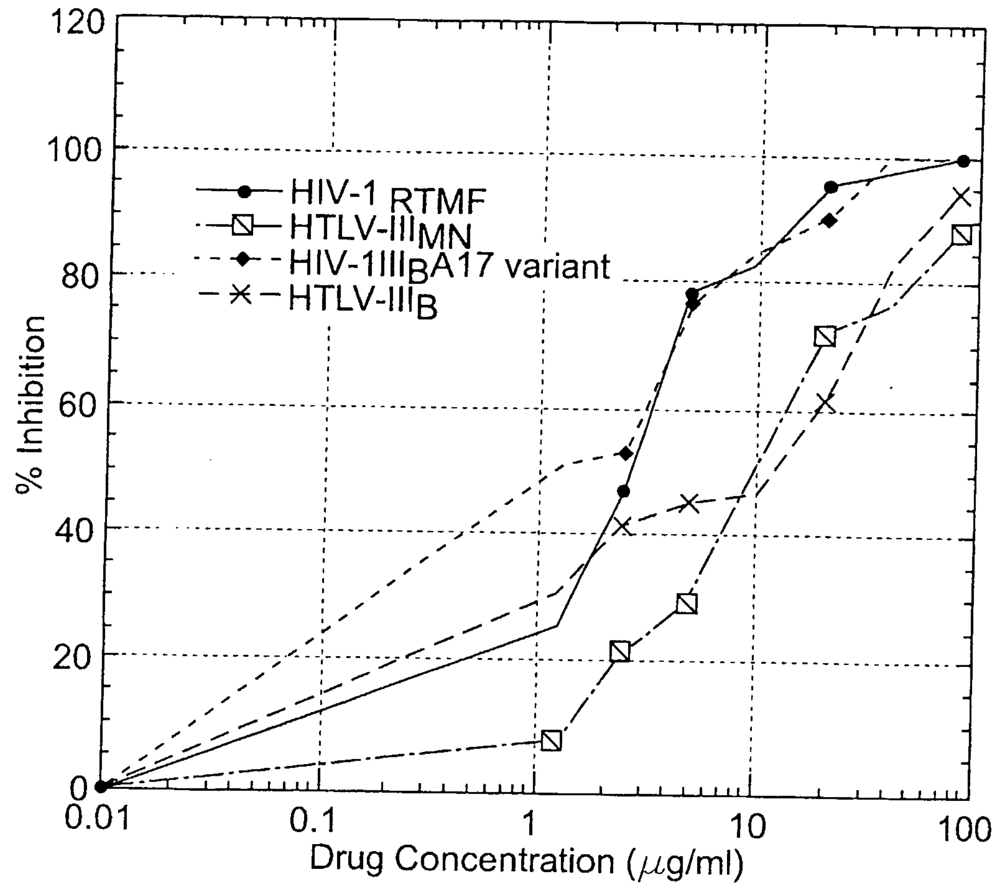

120
100
80
60
40
20
0
% Cell Viability
Control
M522
M532
20
60
100
140
180
Drug Concentration (μg/ml)

FIG. 7B.



120
100
80
60
40
20
0
0.01
0.1
1
10
100
% Inhibition
Drug Concentration (μg/ml)
HIV-1 RTMF
HTLV-IIIMN
HIV-1IIIBA17 variant
HTLV-IIIB

FIG. 8.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/29906

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07D307/86 A61K31/343 A61P31/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07D A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

CHEM ABS Data, WPI Data, BEILSTEIN Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99 66942 A (GEORGETOWN UNIVERSITY, USA) 29 December 1999 (1999-12-29) abstract; claims 4-17 page 25 -page 45; examples	1,8,16, 32
X	H. KOHDA ET AL.: "Isolation of Inhibitors of Adenylate Cyclase from Dan-shen, the Root of Salvia miltiorrhiza" CHEMICAL AND PHARMACEUTICAL BULLETIN, vol. 37, no. 5, 1989, pages 1287-1290, XP002204922 TOKYO JP page 1287, column 2 page 1289, column 2	1,8,32

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 July 2002	26/08/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Paisdor, B

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 01/29906

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	CH. J. KELLEY ET AL.: "Polyphenolic Acids of Lithospermum ruderale Dougl. ex Lehm. (Boraginaceae). 1. Isolation and Structure Determination of Lithospermic Acid." JOURNAL OF ORGANIC CHEMISTRY, vol. 40, no. 12, 1975, pages 1804-1815, XP002204923 EASTON US page 1807, column 1 page 1805, column 1 abstract ---	1,8,32
X	H. YAMAMOTO ET AL.: "Caffeic acid oligomers in Lithosermum erythrorhizon cell suspension cultures" PHYTOCHEMISTRY., vol. 53, 2000, pages 651-657, XP002204924 PERGAMON PRESS., GB ISSN: 0031-9422 page 654 -page 656, column 1 ---	1,8,32
X	R. KASIMU ET AL.: "Comparative Study of Seventeen Salvia Plants: Aldose Reductase Inhibitory Activity of Water and MeOH Extracts and Liquid Chromatography-Mass Spectrometry (LC-MS) Analysis of Water Extracts" CHEMICAL AND PHARMACEUTICAL BULLETIN., vol. 46, no. 3, 1998, pages 500-504, XP002204925 PHARMACEUTICAL SOCIETY OF JAPAN. TOKYO., JP ISSN: 0009-2363 page 503 page 500; examples 1,1A ---	1,8,32
X	PATENT ABSTRACTS OF JAPAN vol. 1998, no. 01, 30 January 1998 (1998-01-30) -& JP 09 241157 A (ALPS YAKUHIN KOGYO KK;NANBA TSUNEO), 16 September 1997 (1997-09-16) abstract ---	1,16
X	T. TANAKA ET AL.: "Four New Caffeic Acid Metabolites, Yunnaneic Acids E-H, from Salvia yunnanensis" CHEMICAL AND PHARMACEUTICAL BULLETIN, vol. 45, no. 10, 1997, pages 1596-1600, XP002204926 TOKYO JP page 1596, column 1; example 1 ---	1,8,32

-/--

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	T. TANAKA ET AL.: "Magnesium and Ammonium-Potassium Lithospermates B, the Active Principles Having a Uremia-Preventive Effect from Salvia miltiorrhiza" CHEMICAL AND PHARMACEUTICAL BULLETIN., vol. 37, no. 2, 1989, pages 340-344, XP002204927 PHARMACEUTICAL SOCIETY OF JAPAN. TOKYO., JP ISSN: 0009-2363 page 342, column 2 -page 343, column 2 page 340; examples 1-3 ---	1,8,32
X	WO 91 19507 A (CEDARS SINAI MEDICAL CENTER) 26 December 1991 (1991-12-26) abstract; claims 1,11,41 ---	1,8,16
X	US 6 043 276 A (H. K. HAN ET AL.) 28 March 2000 (2000-03-28) abstract; claims page 1 -page 4 page 31 -page 38 -----	1,8,16

International application No.
PCT/US 01/29906

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 16-32 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 01/29906

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9966942	A	29-12-1999	US	6037369	A	14-03-2000
			AU	740698	B2	15-11-2001
			AU	4714999	A	10-01-2000
			CN	1312722	T	12-09-2001
			CZ	20004858	A3	12-09-2001
			EP	1089747	A1	11-04-2001
			NO	20006631	A	26-02-2001
			PL	345086	A1	03-12-2001
			TR	200100450	T2	23-07-2001
			WO	9966942	A1	29-12-1999
			US	6043276	A	28-03-2000
JP 09241157	A	16-09-1997	NONE			
WO 9119507	A	26-12-1991	US	5178865	A	12-01-1993
			AP	233	A	07-03-1993
			AU	644669	B2	16-12-1993
			AU	8203891	A	07-01-1992
			CA	2064743	A1	20-12-1991
			EP	0491913	A1	01-07-1992
			JP	5500824	T	18-02-1993
			OA	9651	A	15-05-1993
			WO	9119507	A1	26-12-1991
US 6043276	A	28-03-2000	US	6037369	A	14-03-2000
			EP	1206255	A1	22-05-2002
			WO	0100199	A1	04-01-2001
			AU	740698	B2	15-11-2001
			AU	4714999	A	10-01-2000
			CN	1312722	T	12-09-2001
			CZ	20004858	A3	12-09-2001
			EP	1089747	A1	11-04-2001
			NO	20006631	A	26-02-2001
			PL	345086	A1	03-12-2001
			TR	200100450	T2	23-07-2001
			WO	9966942	A1	29-12-1999